



PHD

**A bacteriological study of Thiobacillus ferro oxidans.**

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A BACTERIOLOGICAL STUDY OF

THIOBACILLUS FERRO OXIDANS

This work was carried out at the Microbiological Research  
Establishment and was supported by the United Kingdom Atomic Energy  
Authority and the Ministry of Defence (Procurement Executive).

Submitted by MARY E. MACKINTOSH, M.Sc.

for the degree of Doctor of Philosophy

of the University of Bath

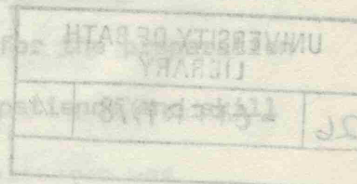
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# A BACTERIOLOGICAL STUDY OF

THIOBACILLUS FERROOXYDANS

Submitted by MARY C. HICKINSMITH, B.Sc.  
for the degree of Doctor of Philosophy  
of the University of Bath  
1977

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## A C K N O W L E D G E M E N T S

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## S U M M A R Y

This work was initiated under a contract with the United Kingdom Atomic Energy Authority to identify the role of Thiobacillus ferro oxidans in the leaching of minerals from low grade ores. A collection of cultures of T.ferro oxidans and other acidophilic thiobacilli, especially T.thio oxidans was made. Samples of soil and pyritic ore and water samples from mine drainage were collected both from this country and abroad from which bacteria were isolated. A method of purifying T.ferro oxidans by plating on a solid medium was developed and the conditions required for growth of colonies from single cells were found. The effect of oxygen on growth is described. This technique also allowed a viable count of the bacteria to be made. Growth rates of T.ferro oxidans on ferrous sulphate media were measured and the effects of aeration, temperature and pH of media on growth were studied. Evidence for strain variation was sought. The production of soluble ferric iron from pyrite and pyritic ores was used to estimate bacterial growth on these substrates. The growth of pure strains of T.ferro oxidans was found to be very uniform for pyrite but mixed thiobacilli cultures were found to solubilise ferric iron from pyrite at a faster rate. The ability of T.ferro oxidans to fix atmospheric nitrogen was discovered and confirmed. Conditions for nitrogen-fixation are described.

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## A Bacteriological study of *Thiobacillus ferro oxidans*

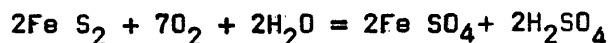
### Introduction

Thiobacillus ferro oxidans is a chemosynthetic, autotrophic, acidophilic bacterium. The ecological importance of the acidophilic thiobacilli was discovered when the problems caused by highly acid drainage from a bituminous coal mine were being investigated. Colmer and Hinkle (1947) showed that although ferrous sulphide was at the root of the problem its oxidation rate in air was too slow to account for the rate at which the acidic ferric sulphate was being produced. The addition of very low concentrations of mercuric chloride, phenol and formaldehyde, all known disinfectants, prevented the reaction as did filtering the mine water through a germicidal pad. If a quantity of the acid mine water was sterilised by filtration and used as a mineral salts medium its ferrous iron content was rapidly oxidised to ferric after inoculation with a sample of unsterilised mine water. The cause of this reaction was identified as a specific bacterium. A second bacterium similar to Thiobacillus thio oxidans (Waksman and Starkey 1922) was also identified which they considered to be involved in the oxidation of sulphur and sulphur compounds to sulphuric acid.

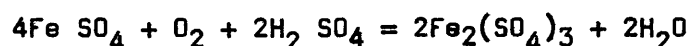
By 1953 the organism involved in the oxidation of ferrous to ferric iron had been identified and was named T.ferro oxidans (Colmer, Temple and Hinkle 1950) and the bacterial involvement in the chemical reactions occurring in acid mine water substantiated. (Temple and Delchamps 1953).



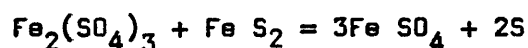
The initial oxidation of ferrous sulphide, e.g. pyrite, may occur chemically but the bio oxidation by T.ferro oxidans greatly accelerates the reaction (Silverman, Rogoff and Wander 1961)



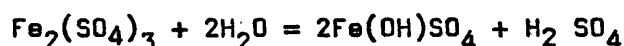
T. ferro oxidans also accelerates the oxidation of ferrous sulphate in acid solution



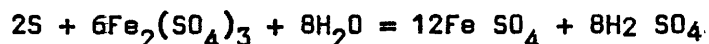
The ferric sulphate formed, being a strong chemical oxidant reacts with more pyrite



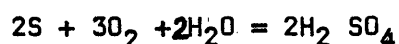
Some ferric sulphate may also be hydrolysed to basic ferric sulphate



The elemental sulphur may be oxidised chemically



but it is more likely to be bacterially oxidised by T.thio oxidans



A cycle is therefore established with the two organisms

T.ferro oxidans and T.thio oxidans which has the net effect of increasing the rate of pyrite oxidation and producing a powerful chemical oxidant, ferric sulphate.

In flooded coal mines the production of acid waters causes pollution and corrosion problems but in other mining situations the acidophilic thiobacilli have proved to be valuable by producing a solution suitable for leaching certain metals from their ores (Duncan, Walden and Trussell 1966). Natural leaching had often

been observed in association with base metal sulphide deposits usually as stream pollution by iron, copper, zinc or just acid. Ore bodies disturbed by mining operations showed an accelerated rate of leaching, an observation made use of since Roman times at the Rio Tinto mines in Spain. In more recent years it has been used in dump, or heap, leaching of copper sulphide minerals. In 1968 100,000 tons per year of copper was being recovered in the Western U.S.A. by irrigating heaps of ore with acid (pH2-3) mine water; the resulting liquors were passed over scrap iron on which the copper was deposited. (Moss and Anderson 1968).

Many important mineral sulphides have been shown to support, or be associated with, microbial activity. Silverman and Ehrlich (1964) list 21 elements so affected. The most important economically are iron, copper, zinc, nickel and cobalt whose sulphide ores can be attacked directly by the bacteria, and uranium where the bacterially produced ferric sulphate oxidises insoluble tetravalent uranium to the soluble hexavalent form.

The genus *Thiobacillus* contains bacteria which are small, gram -ve rod shaped organisms which may be motile. They are frequently found, ecologically, in close association with one another. The characteristic feature of this group is their derivation of energy from the oxidation of one or more reduced or partially reduced sulphur compound including elemental sulphur, sulphides, thiosulphates and polythionates. The final oxidation product is sulphate but sulphur or polythionates may accumulate transiently under certain conditions. *I.ferro oxidans* also utilises ferrous compounds as an electron donor. Most members of this genus are strict autotrophs and derive their carbon from gaseous carbon

dioxide. Some species are capable of assimilating certain organic compounds provided an inorganic electron donor is available.

It has been claimed that some species assumed to be strict autotrophs grow on sucrose under specified conditions. However these observations are not sufficiently well established to be useful as a diagnostic feature (Kelly 1971).

Hutchinson, Johnstone and White (1966) applied a multivariate analysis to the taxonomy of the acidic thiobacilli. They included only those members of the genus which would grow below pH 2.8. They devised a series of tests relating to the growth properties of the organisms and analysed, numerically, the results based on the methods of Beers and Lockhart (1962) and Sneath (1957). They concluded that two groups were undoubtedly present corresponding to the characteristic of T.ferro oxidans on the one hand and T.thio oxidans on the other. The former group contained the iron-oxidising acidophiles and consisted of the essentially similar species T.ferro oxidans, Ferrobacillus ferro oxidans and F.sulfo oxidans. The latter group contained T.thio oxidans and T.concretivorus. They were unable to make a direct comparison with other members of the Thiobacilli as the low pH of the media used inhibited growth in many tests. They showed, however, the importance of initiating growth at the correct pH value especially with thiosulphate media. It was suggested that the name Ferrobacillus ferro oxidans then in use, should be discontinued and all the iron-oxidising acidophiles renamed Thiobacillus spp. since all the strains were capable of oxidising sulphur and thiosulphate in addition to ferrous iron. This nomenclature was subsequently adopted. (Kelly and Tuovinen 1972)



Analysis of the DNA base composition (Jackson, Moriarty and Nicholas 1968) showed three groups within the Thiobacilli which differed in the guanosine + cytosine (G + C) content of their DNA. This grouping substantiated the results of Hutchinson et al, (1966) that T.thio oxidans and T. ferro oxidans are clearly separable and established that T.thio oxidans is not closely related to the rest of the group. The acidophilic thiobacilli come into two different groups. The mole % G + C value for T.thio oxidans was in the 51-52 group and T.ferro oxidans in the 56-57 group. The third group contained organisms in the range 62-68.

In 1973 Agate and Vishiniac characterised the Thiobacillus genus by gas-liquid chromatography of cellular fatty acids. They established profiles of the fatty acid methyl esters (FAME) and showed a sufficient variation in their lipid content to differentiate them into three groups. Group I contained T.ferro oxidans whilst T.thio oxidans was placed in group III which was clearly different from groups I and II.

The recent information on G + C content of DNA and the FAME profiles are useful criteria for the classification of the Thiobacilli. One of the problems which has hindered the classification of these organisms has been purification of strains. Although a solid agar based medium which supports the growth of T.thio oxidans has been known for many years, it was only during the course of the present work that a reliable solid medium was developed which supported the growth of T.ferro oxidans (Tuovinen and Kelly 1973; Mackintosh unpublished).

To complete the relations of T.ferro oxidans three other strains have also been considered for inclusion in the acidophilic thiobacilli. In 1963 a facultative autotroph was described and named Thiobacillus intermedius (London 1963). It was isolated from an enrichment culture of T.thio oxidans growing in liquid thiosulphate mineral medium containing yeast extract (0.005%) as a growth supplement. Subsequent studies showed that T.intermedius at pH2.0-3.0 can grow in a strict mineral medium using reduced inorganic sulphur compounds and it can also grow heterotrophically in a medium containing yeast extract in the absence of an inorganic energy source. Optimal growth is obtained if both reduced sulphur compounds and the organic substrate are provided (London and Rittenberg, 1966).

The second candidate, Thiobacillus perometabolis, was isolated in 1967 (London and Rittenberg 1967). It is very similar to T.intermedius with which it was compared. It required yeast extract for growth but will not grow in the absence of thiosulphate or sulphur.

Guay and Silver (1975) isolated the third candidate which they named Thiobacillus acidophilus from a culture of T.ferro oxidans. It is a facultative autotroph and was isolated by increasing the amount of glucose in the selective medium and decreasing the amount of ferrous sulphate. This organism grows best at pH3.0 and obtains its energy from elemental sulphur or various organic sources including glucose, fructose, sucrose, galactose, etc, but not from ferrous iron or thiosulphate. The G + C ratio for the DNA of

T.acidophilus was 63 mole % and was, therefore, very different from that of T.ferro oxidans (56% G + C) and even more different from T.thio oxidans (51% G + C). The relationship of T.acidophilus and the glucose adapted T.ferro oxidans (Shafia and Wilkinson 1969) was discussed but there was no comparison made between T.acidophilus and T.intermedius. The G + C ratio for T.acidophilus is 63% which is clearly dissimilar to that of other member of the acidic thiobacilli but the G + C ratio for T.intermedius was not determined. The FAME profile for T.intermedius places it in group II (Agate and Vishniac 1973) which is again different from the other acidophilus but a similar profile for T.acidophilus is not available. This evidence, although incomplete, suggested that there is a facultative autotroph which is a distinct third member of the acidophilic thiobacilli. The relationship of the three candidates together with the glucose adapted T.ferro oxidans requires further study to clarify their taxonomic positions.

The bacteriological study of T.ferro oxidans, which is described here, was initiated by a contract with the United Kingdom Atomic Energy Authority who were interested in the fundamental role of bacteria in the uranium leaching process. This study concentrates on the biology of the bacteria rather than the well-investigated chemical reactions carried out by the organisms. The first and most important requirement was for a solid medium for purification of T.ferro oxidans. The growth of various strains was investigated to determine if some were more efficient in leaching minerals than others. To this end a culture collection of strains was made which included strains from the National Collection of Industrial Bacteria and the American Type Culture Collection. Strains were also received from various workers in the field of mineral leaching in



Canada and South Africa who also supplied samples of ore and tailings from which *Thiobacilli* sp. were isolated. Acid mine water, rich in thiobacilli, was received from the National Coal Board. Further strains isolated from samples collected in Great Britain, which are described in 'Collection of cultures', made up the collection. A full list of strains, with origins appears on a later page.

One strain *T.ferro oxidans* Tf3 has been used as a reference strain throughout the work on growth on ferrous sulphate and pyritic ones. The ability of *T.ferro oxidans* to fix atmospheric nitrogen was discovered and is described.

All experimental detail of methods and sources of supply are to be found under 'Methods and Materials'.

## SECTION I

### Collection of Cultures of Thiobacillus sp.

The presence of acidophilic thiobacilli in the soil is exceedingly common and T.ferro oxidans can be isolated from almost any sample which has a close association with iron either as a natural constituent of the soil (as pyrite) or introduced artificially as in iron pipes or drains. There are however, certain environments where the thiobacilli are predominant and present in large numbers forming an important and integral part of the ecology. Such areas are those of mining activity both for metals and for coal where these bacteria flourish on the highly acidic, pyritic substrate associated with these mines. T.ferro oxidans and T.thio oxidans can be isolated (Colmer and Hinkle, 1947) and other thiobacilli may also be present. The role of T.ferro oxidans was discussed in the introduction but the presence and role, if any, of T.thio oxidans is more difficult to understand. Certain mining areas in England and Wales were visited and samples of ore, soil and water collected. It was not possible to carry out an ecological survey of these areas but it was hoped that bacteria isolated from such samples would give an indication of the distributions and variation of strains from different areas and would show any natural diversity which may be lost by continual sub-culturing in a laboratory situation.

### Collection of cultures at Pary's Mountain, Isle of Anglesey

In a natural environment areas of pyritic material that are exposed to rain and water would be colonised by the acidophilic

thiobacilli and the sulphuric acid produced during growth would have a limited local effect but when large areas of pyrite are exposed as in mining operations, open-cast or underground, a very different pattern occurs, the bacteria grow and literally tons of sulphuric acid are produced having a very marked effect upon the environment. Plate 1 shows one such situation. The location is Pary's Mountain in the north-east corner of the Isle of Anglesey. In the foreground can be seen the barren ground, the remains of a derelict building and a flooded pit whilst in the background can be seen the fertile surrounding countryside.

Pary's Mountain was primarily a copper mine but concentrated mineralisation in lead and zinc also occurred. It has been mined since pre-Roman times but the first dated reference records "a great myneral worke" in 1579. In 1768 a great copper deposit was found and the area was then mined extensively until 1833. The Pary's mine on the western side of the mountain was called the "Great Opencast" and it was from this mine that the world price of copper was controlled.

Samples were collected at Pary's Mountain from various sites. Liquid samples in 1oz sterile plastic bottles and rock, soil and mud samples in polythene bags. The samples were kept at room temperature for 6 days before culturing into selective media in the laboratory. Water samples from the lagoon, pH2.0 (Plate 2) and from a flooded mine shaft pH2.4 were rich in T.ferro oxidans and T.thio oxidans which could be seen by microscopic observation. Culturing was required to confirm the identification but the acid pH restricted the growth of many bacteria. The total iron in solution in the lagoon was between 0.27 mg/ml and 0.81 mg/ml. A detailed



PLATE 1

Pary's Mountain, Isle of Anglesey showing spoil tip  
and small area of open-cast mine, now flooded





PLATE 2

Pary's Mountain, Isle of Anglesey showing spoil tip  
and flooded lagoons

analysis of the water was not made. Further samples were collected from a second separate lagoon and from soil heaps adjacent to the open-cast mining area and disused mine shafts. The whole area was sprinkled with mine shafts of unknown depths, many had collapsed and are dangerous. All workings below adit level are flooded.

T.ferro oxidans and T.thio oxidans were isolated from all samples.

#### Collection of cultures in East Devon and West Cornwall

A further study was made in the metalliferous mining area of East Devon and West Cornwall. This covered a large area of varying geology. The important mineral deposits were copper, tin and tungsten, the lodes of which lie in an east-west trend, and lead and zinc minerals which are usually found in north-south trending lodes. As in Anglesey a mining industry flourished in this area long before Roman times. Deep mining did not become viable until the latter part of the 18th century when shafts were sunk and the lodes exploited from these along 'levels' or passages driven along the lode. The finest grade ore was taken to be crushed ready for refining to metal but the poorer grade ore was tipped adjacent to the shaft where much of it remains to-day. Many of the shafts are now flooded.

The most interesting mine visited was the Devon Great Consol which covered a vast area and included many small mines. A Canadian firm was re-processing material from some old tips and access to these areas was restricted. The Devon Great Consol was in its hayday the largest copper mine in Europe. It stopped working in 1902.

Much of the area covered by the mine was barren but in certain areas great efforts had been made to replant with various

types of conifers. Shrubs and bracken had become established. The large barren zones could be divided into two distinct areas; those of derelict buildings and of waste tips. In all areas pyrite, chalcopyrite and arsenopyrite were much in evidence. Among the derelict buildings was the remains of a most impressive furnace where the arsenopyrite was roasted. The walls of the flue, some 30 yards long, were covered with a thick layer of white crystalline arsenic oxide. Some distance away was the chimney (Plate 3). A waste tip rich in arsenopyrite is seen in front of the chimney. (A few conifers are also seen). Samples were collected from the tip and despite the presence of arsenic were rich in I.ferro oxidans and I.thio oxidans.

The waste tip which we were allowed to visit was of typical appearance (Plate 4). Although there was no surface water it had been raining and the tip was damp. Using pH indicator papers a figure of pH 1.7 - pH 2.0 was found wherever tested on the tip. The rocks and rubble had a brown, distinctly vitreous appearance. When touched small pieces of rock crumbled exposing a mass of rust-coloured debris, large conglomerates fragmented at the tap of a hammer. In the laboratory bacteria could be obtained from the surface of samples collected at this tip, simply by washing and I.ferro oxidans and I.thio oxidans were present in large numbers.

The waste tips at the Devon Great Consol mine are being worked but at other mines, Drakewall and Gunnislake, the waste tips have been sold for 'road rubble' and locally for domestic garden paths which they claim "will remain weed-free". Since sulphuric acid leaches out every time it rains they will certainly remain weed-free, but if there are piped services running underneath such paths there will be future problems.





PLATE 3

Devon Great Consol Mine. Large spoil tip rich  
in pyrite, chalcopyrite and arsenopyrite.



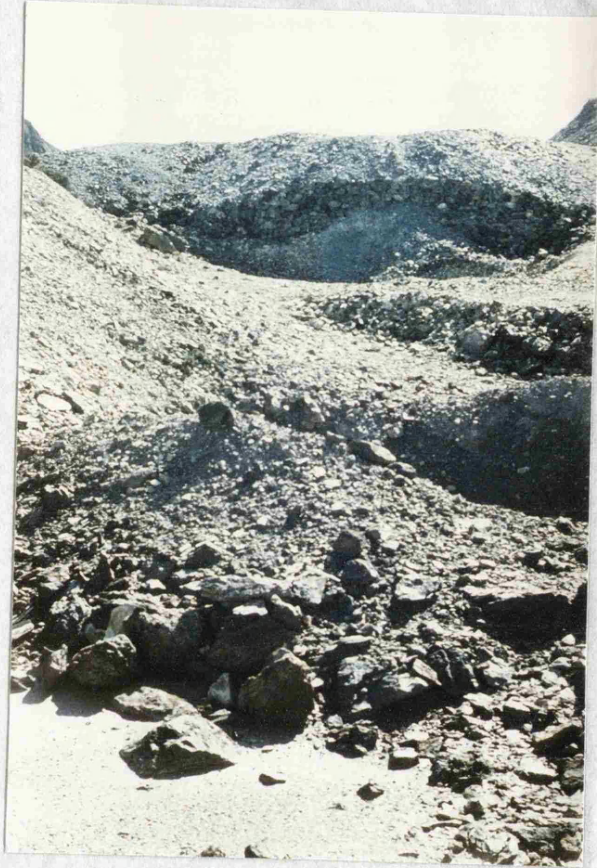


PLATE 4

Devon Great Consol Mine. Spoil tip, pH 1.8 - 2.0

A much smaller mine at Mary Tavey was visited. It was a lead-zinc working by open-cast and shaft mining very near a small river. There was a characteristic lack of vegetation at the site and evidence of galena and chalcopyrite. On many rocks and stones yellow sulphur deposits could be seen. Samples were collected. The mine adit which was some 900 yards downstream was producing very clear water of pH 6.4. Around the opening was a deposit of iron, possibly ferric hydroxide. A water sample from the adit and rock samples from the mine all contained T.ferro oxidans and T.thio oxidans. This site was particularly rich in T.thio oxidans.

Gawton mine was also visited and samples collected.

Two geologically different mines visited were at Kit Hill and Birch Tor. These both represent mine workings in granite. Kit Hill is an exposed granite mass overlooking the Tamar Valley at the top of which was the mining area which was of shallow tin workings. The old building and shafts were fenced off but the surrounding area was grass covered. The mine adit was some distance down the hill and gave no evidence of iron precipitation. A pool had formed and was quite clear pH 6.4. T.ferro oxidans was, however, isolated from a sample of the water. Unfortunately the area of the mine was so dangerous that no other samples could be collected. Birch Tor was on central Dartmoor. It was an old open-cast tin mine within Dartmoor granite. The tin mined was of metallic oxide rather than the sulphide and thiobacilli were not isolated from any sample collected at this site.

#### Collection of cultures from coal mines

As T.ferro oxidans and T.thio oxidans were originally isolated from acid mine waters from a coal mine (Colmer and Hinkle 1947)

it would be the most likely place to find them. Samples of acid mine water were received from the Scottish Regional Laboratory of the National Coal Board in Edinburgh. The 12 samples represented different areas of collection in two mines and from spoil tip drainage. The samples had a pH of 2.8 - 3.0 and the total iron varied from 0.816 mg/ml to 2.1 mg/ml with most samples containing about 1.6 mg/ml. All samples contained T.ferro oxidans and T.thio oxidans.

Samples collected from the coal mining area in the Forest of Dean also contained thiobacilli but selection was made for samples containing coal, pyritic material or iron deposit.

#### Collection of cultures from pyritic soil

The biological oxidation of pyrite in soil was causing acidic soils in the newly reclaimed marshland of the Zuidersea (Quispel, Harmsen and Otzen, 1952). T.thio oxidans and other thiobacilli were found to stimulate the reaction. In this country more recently a similar problem has arisen in the under drainage of pyritic Fenland soils; the drains becoming blocked by ochreous sludge. Soils of marine origin contain significant amounts of pyrite and when drained oxidation of the pyrite occurs, sulphuric acid is produced and the soils become very acid, pH 2.2 (Bloomfield, 1972). The ochre is caused by filamentous iron bacteria Gallionella sp. which secretes a non-living, branching, spirally-twisting stalk composed of precipitated ferric hydroxide, and Thiobacillus sp. which produce the acid and precipitate basic ferric sulphate.

Of 6 samples received from an experimental draining scheme of pyritic soil in Cambridgeshire, T.ferro oxidans and T.thio oxidans were isolated from all samples.

It should not be assumed that T.ferro oxidans and T.thio oxidans are the only bacteria present in a mixed acidophilic culture that would be obtained from a sample collected in a natural environment. T.intermedius and T.acidophilus may often be present and grow autotrophically in the presence of sulphur. They were not looked for in this study; T.acidophilus only being described in 1975. What role they play in the complex energy cycle from pyrite has yet to be determined. Even that of T.thio oxidans is not understood. Leathen, Brayley and McIntyre (1953) found no enhancement of acid formation by T.thio oxidans from sulphuritic material, with the exception of marcasite, but suggested that some crystalline forms of  $\text{FeS}_2$  may be susceptible to oxidation by it to account for its constant presence in mine effluents. Silverman, Rogoff and Wender (1961) confirmed these findings and claimed that T.thio oxidans appeared to have no role in pyrite oxidation. This may be so but the presence of T.thio oxidans is too consistent for it to be simply using up free sulphur formed during pyrite oxidation by T.ferro oxidans.

#### Glucose utilisation by T.ferro oxidans

The ability of T.ferro oxidans to grow on organic carbon was reported by Shafia and Wilkinson (1969). Following their technique various strains including Tf3 were adapted to grow heterotrophically on glucose. When the ferrous sulphate was omitted, however, only strains Tf3 and Tf6 grew satisfactorily. The bacterial cells in the other cultures were so morphologically different from the original strains that they were discarded.

Tf3g (glucose adapted strain) was grown in continuous culture in a medium of 'F' basal salts (described in section II) with

1% w/v glucose and 0.1% w/v yeast extract pH 2.2 for two months. Growth was good and a cell density of  $3 \times 10^8$  cell/ml was maintained if the medium was pH 2.2, aeration of the 500ml pot did not exceed 0.15 litres/minute (which is very low) and yeast extract was present. The generation time was 11 hours. At weekly intervals a sample was taken, washed to remove the glucose medium and used to inoculate either a liquid ferrous sulphate medium, to see if iron was still oxidised, or a nutrient laboratory medium to see if the culture was contaminated. After 5-7 days incubation at 30°C the ferrous sulphate medium showed the brown precipitation indicative of growth, and microscopic examination showed the characteristic bacteria of T.ferro oxidans. No contamination was found. Even after two months growth in continuous culture the bacteria were still able to oxidise iron.

The position of the glucose adapted T.ferro oxidans was questioned when Guay and Silver (1975) isolated T.acidophilus. The relationship of these two organisms is discussed in the introduction. The adapted strain of Shafia and Wilkinson (1969) did not lose its iron-oxidising ability but the T.ferro oxidans adapted to grow on glucose by Tabita and Lundgren (1971) did. An explanation could be the purity of the original T.ferro oxidans strain. If T.acidophilus, or a similar type of organism, was present in the original culture it would grow on the glucose before T.ferro oxidans had time to adapt and would be misidentified as a glucose adapted strain. As the purity of the glucose utilising strain increased with subculturing so would the iron oxidising ability of the culture decrease. In a continuous culture an unadapted, non-replicating T.ferro oxidans could not have remained in the culture in the total absence of an energy source for

more than a few days and the ability of Tf3g to oxidise iron after two months continuous culture strongly suggests that T.ferro oxidans can be adapted to grow heterotrophically.

The purity of Thiobacillus sp. cultures must be seriously considered because some strains of T.ferro oxidans received from National Collection both from this country and America were mixed cultures, T.thio oxidans being the most usual contaminant.

#### Maintenance of cultures

The maintenance of T.ferro oxidans has been by subculturing and although this need only be done every 3-4 months if a 200ml stock culture is kept on ferrous sulphate medium it becomes tedious if any number of strains are kept. Freeze drying killed all the cells from an initial culture of  $3.4 \times 10^{10}$  cells/ml.

Thiobacilli will survive in sulphuric acid even at concentrations in which they are unable to grow, i.e. below pH 1.0 (Kempner, 1966). Initially stock cultures were kept in sulphur medium (Hutchinson, Johnstone and White, 1966). After 8 days growth at 30°C excess sulphur was removed by filtration through Millipore filters, 8 $\mu$  and 3 $\mu$  pore size. 2ml of the sulphur-free culture was placed in a sterile ¼oz bijou bottle, the cap screwed on tightly and the bottle kept at room temperature. Under these conditions, as long as evaporation did not occur the bacteria, both T.ferro oxidans and T.thio oxidans remained viable for at least 2 years.

Recently the technique of palleting T.ferro oxidans in liquid nitrogen (Manchee, 1975) was found to be very successful with an 0.1% survival.



Strains of Thiobacilli sp. in the Culture Collection

<u>MRE no.</u>	<u>STRAIN</u>	<u>ORIGIN</u>
Tf2	<u>T.ferro oxidans</u> NC1B 8451	J.Postgate - originally from the National Chemical Laboratory, Teddington
Tf3	<u>T.ferro oxidans</u> (Reference strain)	J. Yates - ARC Nitrogen fixation Unit, University of Sussex.
Tf4	<u>T.ferro oxidans</u> British Colombia Research (BCR) strain	British Colombia Research Council, Vancouver 8, Canada.
Tf5	<u>T.ferro oxidans</u> "Ottawa" strain	Department of Energy, Mines and Resources, Mines Branch, Ottawa, Canada.
Tf6	<u>T.ferro oxidans</u> + ? mixed culture	Stope floor - Mine at Elliot Lake, Canada.
Tf7	<u>T.ferro oxidans</u> NC1B 8455 )	National Collection of Industrial Bacteria Torry Research Station, Aberdeen.
Tf8	" NC1B 9490 )	
Tf9	" NC1B 10435 )	
To10	<u>T.thio oxidans</u> NC1B 8342	J.Postgate - originally from the National Chemical Laboratory, Teddington.
Tf11	<u>T.ferro oxidans</u> + ? mixed culture	Stagnant pool at Elliot Lake Mine, Canada.
Tf12	" "	Fresh pool at Elliot Lake Mine, Canada
Tf17	<u>T.ferro oxidans</u> + ? mixed culture	D. Wakerly - Warren Spring Laboratory, Stevenage
Tf18	<u>T.ferro oxidans</u>	D. Wakerly - growing at pH 1.5 on pyrite at Warren Spring Laboratory, Stevenage.
Tf19	<u>T.ferro oxidans</u> ATCC 13661 )	American Type Culture Collection 12301 Parklawn Drive, Rockville, Maryland, U.S.A.
Tf20	" ATCC 13598 )	
Tf21	" ATCC 13728 )	
Tf22	" ATCC 19859 )	
T 23	<u>Ferrobacillus sulfo oxidans</u> ATCC 14119 )	American Type Culture Collection
T24	<u>T.concretivorus</u> ATCC 19703 )	

<u>MRE no.</u>	<u>STRAIN</u>	<u>ORIGIN</u>
Tf26	<u>T.ferro oxidans</u> Research Strain	Chamber of Mines South Africa CMSA/1
Tf27	<u>T.ferro oxidans</u> + ? mixed culture )	
Tf28	" "	Slimes Dam sample South Africa
Tf29	" "	
To30	<u>T.thio oxidans</u>	From Tf29
Tf31	<u>T.ferro oxidans</u>	Elliot Lake ore sample washings
Tf32	"	" " " 'fines'
To33	<u>T.thio oxidans</u> NCIB 8342 )	
To34	" NCIB 8343 )	
To35	" NCIB 8444 )	National Collection of Industrial Bacteria
To36	" NCIB 9112 )	
Tf47	<u>T.ferro oxidans</u>	BCR - new culture received from D.W. Duncan
Tf48	<u>T.ferro oxidans</u> (SR1) mixed culture )	
Tf49	" (SR2) " "	Samples from J.M. Garrett - National Coal Board - from acid mine water
Tf50	" (SR3) " "	from two mines in Scotland
Tf51	" (SR4) " "	
Tf52	" (SR5) " "	
Tf53	" (SR11) " "	
Tf54	" (SR12) " "	Sub cultures in 'S' medium of mine water
Tf55	<u>T.ferro oxidans</u> + mixed culture	W.A. Gow - Mine tailing, Ottawa, Canada

<u>MRE no.</u>	<u>STRAIN</u>	<u>ORIGIN</u>
Tf480	<u>I.ferro oxidans</u>	Isolated from Tf48 (SR1)
Tf490	<u>I.ferro oxidans</u>	" " Tf49 (SR2)
Tf500	<u>I.ferro oxidans</u>	" " Tf50 (SR3)
Tf510	<u>I.ferro oxidans</u>	" " Tf51 (SR4)
Tf520	<u>I.ferro oxidans</u>	" " Tf52 (SR5)
To70	<u>I.thio oxidans</u>	Isolated from 'F' culture of Tf50(SR3)
To701	<u>I.thio oxidans</u>	" " 'S' " Tf50(SR3)
To72	<u>I.thio oxidans</u>	" " 'F' " Tf52(SR5)
To721	<u>I.thio oxidans</u>	" " 'S' " Tf52(SR5)
To734	<u>I.thio oxidans</u>	" " 'F' " Tf54(SR12)
Tf74	<u>I.ferro oxidans</u> + mixed culture	Tharsis pyrite
Tf740	<u>I.ferro oxidans</u>	From Tf74
To75	<u>I.thio oxidans</u>	From Tf74
Tf3g	<u>I.ferro oxidans</u> (glucose adapted)	Tf3 adapted to grow on glucose.
T.PM	Thiobacillus <u>sp.</u>	Mixed cultures isolated from Pary's Mountain (nos. 1 - 12)
T.MT	Thiobacillus <u>sp.</u>	Mixed cultures isolated from MaryTavey Devon (nos. 1 - 7)
T.DGC	Thiobacillus <u>sp.</u>	Mixed cultures isolated from the Devon Great Consol Mine, Devon (nos. 1 - 10)
T.GW	Thiobacillus <u>sp.</u>	Mixed cultures isolated from the Gawton Mine Devon (nos. 1 - 4)
T.KH	Thiobacillus <u>sp.</u>	Mixed cultures isolated from Kit Hill adit Devon (nos. 1 - 2)
T.CPF	Thiobacillus <u>sp.</u>	Mixed cultures isolated from drains and soil of pyrite fen soil in Cambridgeshire (nos. 1 - 6)
T.FD	Thiobacillus <u>sp.</u>	Mixed cultures isolated from soil and tips of a disused coal mine in the Forest of Dean (nos. 1 - 3)

## SECTION II

### Growth of *T.ferro oxidans* on a solid medium

A solid medium able to support the growth of *T.ferro oxidans* when using ferrous sulphate as its energy source was needed for the purification of strains and for the estimation of cell numbers in a liquid  $\text{FeSO}_4$  culture by a viable count. *T.ferro oxidans* will grow on agar when sodium thiosulphate is the source of energy. The pH of this medium is pH 5.5. When ferrous sulphate is provided as the source of energy the pH is far more acid, pH 2.0 - 3.0. Agar is an acidic polysaccharide extracted from certain seaweeds. Although it consists primarily of galactose with occasional sulphate residues, because of its natural origin agar contains various metabolites. Agar is reported to inhibit colony development of *T.ferro oxidans* on ferrous iron medium (Bryner and Jameson, 1958; Unz and Lundgren, 1961; Beck, 1967) and it may be that the acid pH of the medium causes a release of toxic material from the agar.

Various agars were tried to see if they inhibited growth; Difco Bacto Agar, Oxoid Agar No. 1 and Davies' New Zealand Agar. The mineral salts used with the agar was the basal salts of Leathen, Kinsel and Braley (1956) which is called 'F' salts, to which sterile ferrous sulphate solution was added to give a final concentration of 5 mg/ml Fe. This is called 'F' medium and only modifications of this basic recipe, if used, will be given more fully in the text. Full details of all standard media used and methods of preparation are given in the section "Methods and Materials".

Seven strains of T.ferro oxidans including the reference strain Tf3 were streaked on to the different agar plates and incubated for 20 days at 30°C. No growth was observed on any plate. The method of Meynell and Meynell (1965) for purifying agar by washing was tried. This had no effect on growth so an alternative to agar as a solidifying agent was sought.

#### Silica gel as a solidifying agent

Silica gel (Leathen, McIntyre and Braley 1951) was tried with mixed success. It was tedious to prepare and basically unsatisfactory as the gel was not always stiff enough to allow streaking. Its use was discontinued.

#### Agarose as a solidifying agent

Agarose is a non-ionic, sulphate-free fraction of agar. It is a linear polysaccharide with alternate residues of D-galactose and 3,6, anhydro-L-galactose units. It is of a much higher purity than agar but should have similar solidifying properties. The sources of agarose used were Seravac Laboratories, L'industrie Biologique Francaise S.A. and British Drug House.

A series of concentrations of agarose in water were made up and sterilised to determine a suitable working strength. Plates were poured and after allowing 1 hour to solidify, each concentration was tested to check that it had solidified and if so, was firm enough for streaking with a nickel/chrome wire loop. The results are shown in Table 1. A working strength of 0.8% was firm enough for all standard bacteriological techniques but not too firm for pouring when molten. For overlay technique a

concentration of 0.8% could also be used. Since agarose was more expensive than agar 5.5 cm petri dishes were used instead of the standard 9.5 cm dishes, thus reducing the medium required per plate from 20 ml to 3 ml.

TABLE 1

Effect of various concentrations of agarose for solidifying ferrous sulphate medium for bacteriological work

<u>Concentration of Agarose</u> % (w/v)	<u>Solidification</u>	<u>Sensitivity to streaking</u>	
		<u>Platinum loop</u>	<u>nickel/chrome loop</u>
1.0	+++	+++	+++
0.8	++	++	++
0.5	++	++	+
0.3	+	+	-
0.15	-	-	-

+++ very firm

+ soft

++ firm

- unusable

Effect of pH on agarose medium

The agarose was made up at twice the required working strength (1.6%) in water and autoclaved. It was mixed with an equal volume of double strength 'F' salts at pH 1.5, pH 2.0 and pH 3.0. Ferrous sulphate solution was added and the plates poured. All solidified well. The medium at pH 3.0 was distinctly brown after incubation for 2 days at 30°C; the two of lower pH were not. A pH of 2.0 was chosen for routine work. If the agarose was made

up with the mineral salts at an acid pH and autoclaved, acid hydrolysis of the agarose occurred and the subsequent plates poured failed to solidify.

#### Growth on agarose-ferrous sulphate medium

Strains of T.ferro oxidans, which had been grown in 'F' medium, were tested for their ability to form colonies on the 'F' agarose medium. Plate 5 shows the results of streaking three strains of T.ferro oxidans on an 'F' agarose plate and incubating at 30°C for 14 days. A deep brown precipitate was seen in the agarose with no evidence of colony growth on the surface. The precipitate was the result of bacterial activity as it was not produced by streaking uninoculated 'F' medium or a 'millipore' filtrate (0.45µ pore size) of a culture. This result suggested that T.ferro oxidans might be microaerophilic and capable of growing in the medium if not on it. A series of 'F' agarose plates were set up, inoculated at various dilution with T.ferro oxidans by the spreading technique and the plates incubated in an atmosphere of reduced oxygen tension. The atmosphere was achieved in a glass anaerobic jar. The plates were placed in the anaerobic jar which was evacuated by a water pump and the air replaced with nitrogen + 5% carbon dioxide (supplied by BOC). The plates, in the jar, were incubated at 30°C. After 10 days incubation distinct colonies could be seen. The colonies appeared brown and each was surrounded by a dark brown precipitate of basic ferric sulphate as illustrated in Plate 6. To confirm that these features were in fact viable colonies some 30 isolated 'colonies' were tested. Each was stabbed with a wire which was used to inoculate 3 ml of liquid 'F' medium. The cultures were incubated and all subsequently grew showing the





PLATE 5

Effect of streaking T.ferro oxidans on 'F' agarose medium.  
The plate was incubated at 30°C for 14 days.

0.875 x actual size

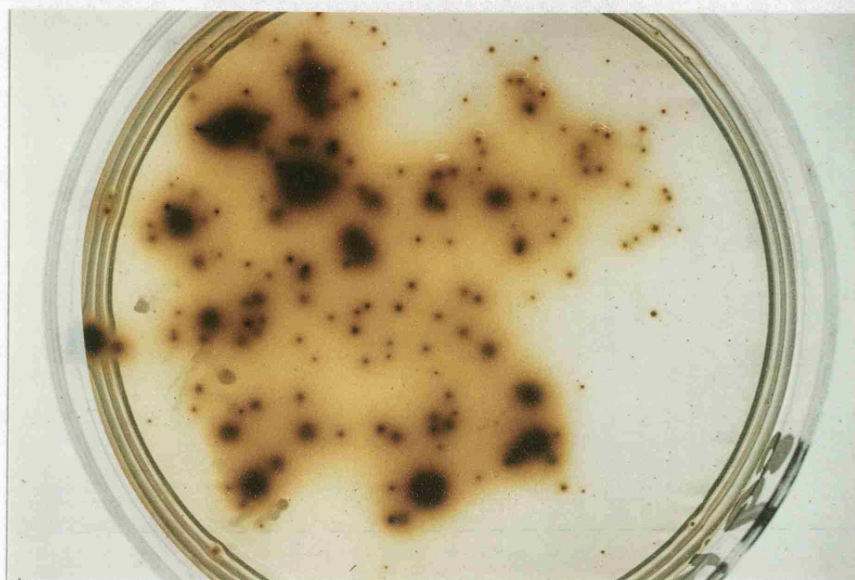


PLATE 6

Colony formation of T.ferro oxidans on 'F' agarose  
incubated under  $N_2$  + 5%  $CO_2$  at  $30^{\circ}C$  for 10 days

magnification x 1.1

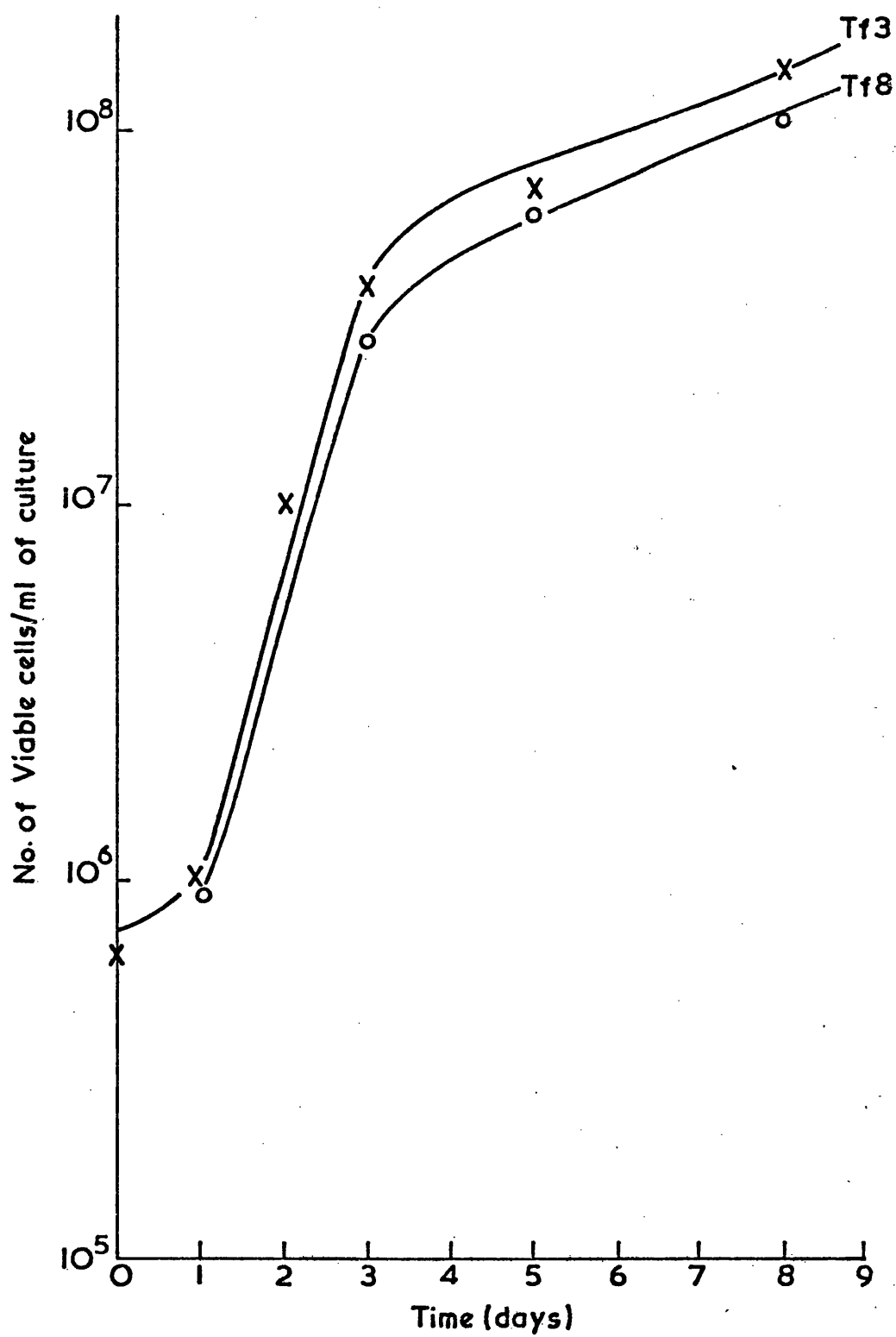
characteristic bacteria of T.ferro oxidans and brown precipitate in the medium after 7 days incubation at 30°C. All the strains of T.ferro oxidans which were tested grew and formed colonies. No difference was found in the growth supporting ability of the different brands of agarose and therefore BDH agarose was chosen for further work as it was the most readily available.

#### Growth of T.ferro oxidans as estimated by a viable count

Typical growth curves for T.ferro oxidans strains Tf3 and TfB as estimated by a viable count are shown in Figure 1. Each strain was grown in 150 ml of 'F' medium in a 500 ml flask which was incubated static at 30°C. The inoculum of 1 ml was taken from a 4 day culture growing in the same medium. The plate counts were made at daily intervals. A 1 ml sample was taken at 14.00 on each day. Each sample was diluted as required in 'F' salts and plated by the pour plate method (1.5 ml of 'F' agarose medium being used for the overlay). The plates were incubated in glass anaerobic jars. A pad of filter paper, about 10. cm diameter, which was saturated with sterile distilled water was placed at the bottom of each jar to prevent dehydration of the plates during incubation. The jars were evacuated and then filled with nitrogen plus 5% carbon dioxide and this procedure was repeated once. A separate jar was used on each day that samples were taken so that the atmosphere was not disturbed during incubation. Plates were incubated at 30°C for 10 days at which time the colonies were counted and the graphs plotted. The mean generation time for growth in 'F' medium was found to be 10-12 hours.

The viable count was used to confirm that 0.8% agarose and pH2-2.2 were the most favourable conditions for colony formation.

FIG.1

Growth of T.ferrooxidans in Ferrous sulphate medium

### Effect of atmosphere on colony formation on 'F' agarose medium

To study the effect of various atmospheric conditions on colony formation of T.ferro oxidans slide cultures were used (Postgate, Crumpton and Hunter 1961). A slide culture is made by placing a metal annulus, 22mm diameter and 1mm thick, on a microscopic slide 0.8-1.0mm thick and filling the chamber formed by the annulus with 0.25 ml of molten agarose medium. The agarose adheres to the ring and to the glass. Two such chambers could be placed on one slide. The slides and annuli were kept in ethanol and sterilised by flaming prior to use. The bacterial culture for inoculation was diluted to approximately  $10^7$  bacteria/ml and spread with a platinum loop on to the surface of the agarose. The chamber was not sealed by a glass coverslip but the slide was placed in a petri dish on a pad of water-saturated filter paper. The petri dishes with their culture chambers were incubated in anaerobic jars in atmospheres either of  $N_2 + 5\% CO_2$  or air + 5%  $CO_2$ . After one and two day incubation the culture chambers were allowed to equilibrate to room temperature in the petri dishes before inspection with a phase-contrast microscope using a green light.

Table 2 shows the growth of three strains of T.ferro oxidans on 'F' agarose chamber incubated in  $N_2 + 5\% CO_2$  or air + 5%  $CO_2$ . The chambers were inspected at 24 hours and 48 hours and were scored for single cells which had not started to grow groups of cells which had undergone 1 or 2 divisions only and those which had divided to form microcolonies where the number of cells present could not be counted. Microcolonies seen at 24 hours were considered

to be groups of cells which had been simultaneously deposited during inoculation. It was possible to inspect the chambers daily for 4-5 days until either the growing colonies produced so much precipitation that microscopic observation was impossible or (after 5 days) the chambers had dried out, despite attempts at humidification.

TABLE 2

Effect of atmosphere of incubation on colony formation by T.ferro oxidans on ferrous-sulphate agarose slide cultures at 24 and 48 hours.

<u>Strain</u>	<u>Atmosphere</u>	<u>% single cells</u>	<u>% 2-4 cell groups</u>	<u>% micro colonies</u>	<u>time of incubation at 30°C</u>
Tf3	N <sub>2</sub> +5%CO <sub>2</sub>	44	55	2	24 hours
Tf2		47	52	1	
Tf18		36	63	1	
Tf3		21	1	78	48 hours
Tf2		23	2	75	
Tf18		5	6	86	
Tf3	air+5%CO <sub>2</sub>	76	23	1	24 hours
Tf2		81	18	1	
Tf18		65	35	2	
Tf3		40	56	4	48 hours
Tf2		70	27	3	
Tf18		47	51	2	

The results in Table 2 confirm the beneficial effect on colony formation of incubation in N<sub>2</sub>+ 5% CO<sub>2</sub> and show that after 48 hours

78% of Tf3 bacteria had formed microcolonies. Further observations showed that cells which had not divided in 48 hours did not do so during the observable time of the experiment. When the slide cultures were incubated in air + 5% CO<sub>2</sub> initially division occurred but only 4% of the cells progressed beyond the 4 cell stage and, of these, none grew beyond microcolonies.

No consistent variation among strains was found although the % viability under N<sub>2</sub> + 5% CO<sub>2</sub> varied from as little as 1% to as much as 98% in different experiments. The reason for this variation was eventually found and will be discussed below.

Effect of atmosphere on colony formation on thiosulphate agarose medium.

The thiosulphate medium (ST) used was that of Hutchinson, Johnstone and White (1966) solidified with 0.8% agarose. The slide chambers were set up and inoculated from liquid thiosulphate grown cultures and incubated at 30°C under N<sub>2</sub> + 5% CO<sub>2</sub> or air + 5% CO<sub>2</sub>. The cultures were inspected at 24 and 48 hours and the results are given in Table 3. The atmosphere had little effect during the first 24 hours but by 48 hours growth under N<sub>2</sub> was greatly retarded and no microcolonies were found. In air growth progressed to form such colonies with viabilities between 7% and 51%. This result shows that T.ferro oxidans has a greater demand for oxygen when growing on thiosulphate than on ferrous sulphate medium.



TABLE 3

Effect of atmosphere of incubation on formation of colonies of T.ferro oxidans on thiosulphate agarose slide cultures at 24 and 48 hours.

<u>strain</u>	<u>atmosphere</u>	<u>% single cells</u>	<u>% of 2-4 cell groups</u>	<u>% micro colonies</u>	<u>time of incubation at 30°C</u>
Tf3	N <sub>2</sub> +5%CO <sub>2</sub>	33	65	1	24 hours
Tf2		27	62	1	
Tf18		35	63	2	
Tf3		11	88	1	48 hours
Tf2		9	90	1	
Tf18		15	83	2	
Tf3	air +5%CO <sub>2</sub>	25	73	2	24 hours
Tf2		38	61	1	
Tf18		38	59	3	
Tf3		20	54	26	48 hours
Tf2		31	62	7	
Tf18		23	26	51	

Colony morphology on ferrous-sulphate and thiosulphate medium

A very distinctive morphological difference was observed in colonies grown on 'F' agarose medium and 'ST' agarose medium as is illustrated in Plates 7 and 8. Both photographs are of Tf3.

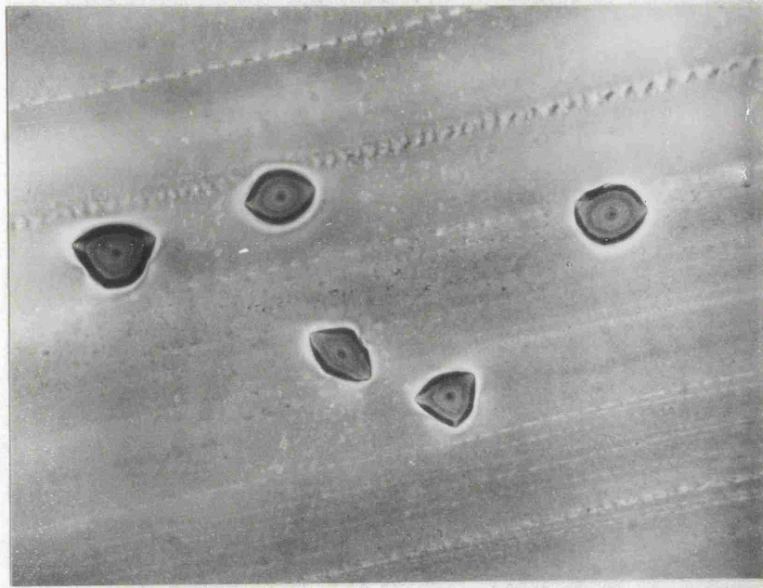


PLATE 7

Colonies of T. ferro oxidans Tf3 on 'F' agarose incubated  
under  $N_2 + 5\% CO_2$  at  $30^{\circ}C$  for 9 days.

magnification x 60

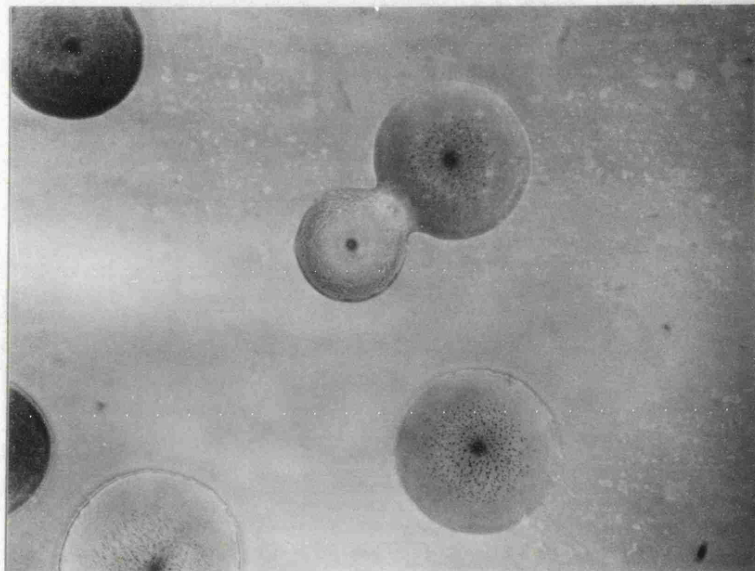


PLATE 8

Colonies of T. ferro oxidans Tf3 of 'ST' agarose incubated  
under air +  $5\% CO_2$  at  $30^{\circ}C$  for 9 days.

magnification x 60

Plate 7 shows the colonies formed of 'F' agarose after 9 days incubation at 30°C under  $N_2 + 5\% CO_2$ . The colonies appear dark brown at the base but much lighter in colour at the crown. The tricorn appearance is very characteristic and was found with all strains of T.ferro oxidans growing on 'F' agarose. Plate 8 is Tf3 growing on 'ST' agarose after 9 days at 30°C under air + 5%  $CO_2$ . The colonies are cream in colour.

#### Agarose versus agar as solidifying agent

As the critical factor for colony formation had been shown to be the atmosphere in which the cultures were incubated, the inhibition of growth by agar was looked at again. The results in Table 4 show that if air is replaced by nitrogen during incubation there was very little difference in the % viability of cells grown on 'F' agar or 'F' agarose. There was greater variation between different brands of agar.

Agar contained many more impurities and even when washed the agar medium was more opaque which made microscopic observation more difficult.

TABLE 4      Effect of atmosphere of incubation and solidifying agent on colony formation of T.ferro oxidans Tf3 as estimated by % viability after 48 hours.

<u>Solidifying agent</u>	<u><math>N_2 + 5\% CO_2</math></u>	<u>air + 5% <math>CO_2</math></u>
agarose BDH	85.7%	0
Difco Bacto Agar	71.8%	0
Oxoid No. 1 Agar	81.6%	0

Incubated at 30°C

Ferrous sulphate medium

### Factors affecting the plating efficiency of 'F' agarose medium

Variation in the percentage viability of T.ferro oxidans when growing on 'F' agarose under  $N_2 + 5\% CO_2$  was found to be related to the phase of growth of the liquid culture from which the sample for the inoculation of the agarose was taken. During growth of Tf3 in liquid 'F' medium samples were taken, as previously described, and plated for a viable count and an aliquot of the same sample was used to inoculate a slide culture. The % viability after 48 hours incubation under  $N_2 + 5\% CO_2$  at  $30^\circ C$  was found. The results are given in Fig.2 which shows that during the lag phase of growth about 7% of colonies plated grew to form colonies. This number increased to 20% in the early phase of logarithmic growth and to 90% during the true logarithmic growth phase. As the stationary phase approached the % viability dropped to 40% and during the stationary phase it dropped still further. After 5 weeks of standing at room temperature the % viability was less than 0.1% but even after 2 years the culture was still viable.

### Effect of change of media on colony formation

When the energy source for growth of T.ferro oxidans was changed it was accompanied by a drop in % viability on solid medium as shown in Table 5. All samples for inoculation of the slide chambers were taken from liquid cultures in the logarithmic phase of growth. Sulphur for the sulphur medium (Hutchinson, Johnstone and White 1966) is provided as colloidal sulphur and is not adaptable to a solid medium.

FIG.2.

Growth of Thiobacillus ferrooxidans Tf 3 at 30°C in  
ferrous sulphate medium

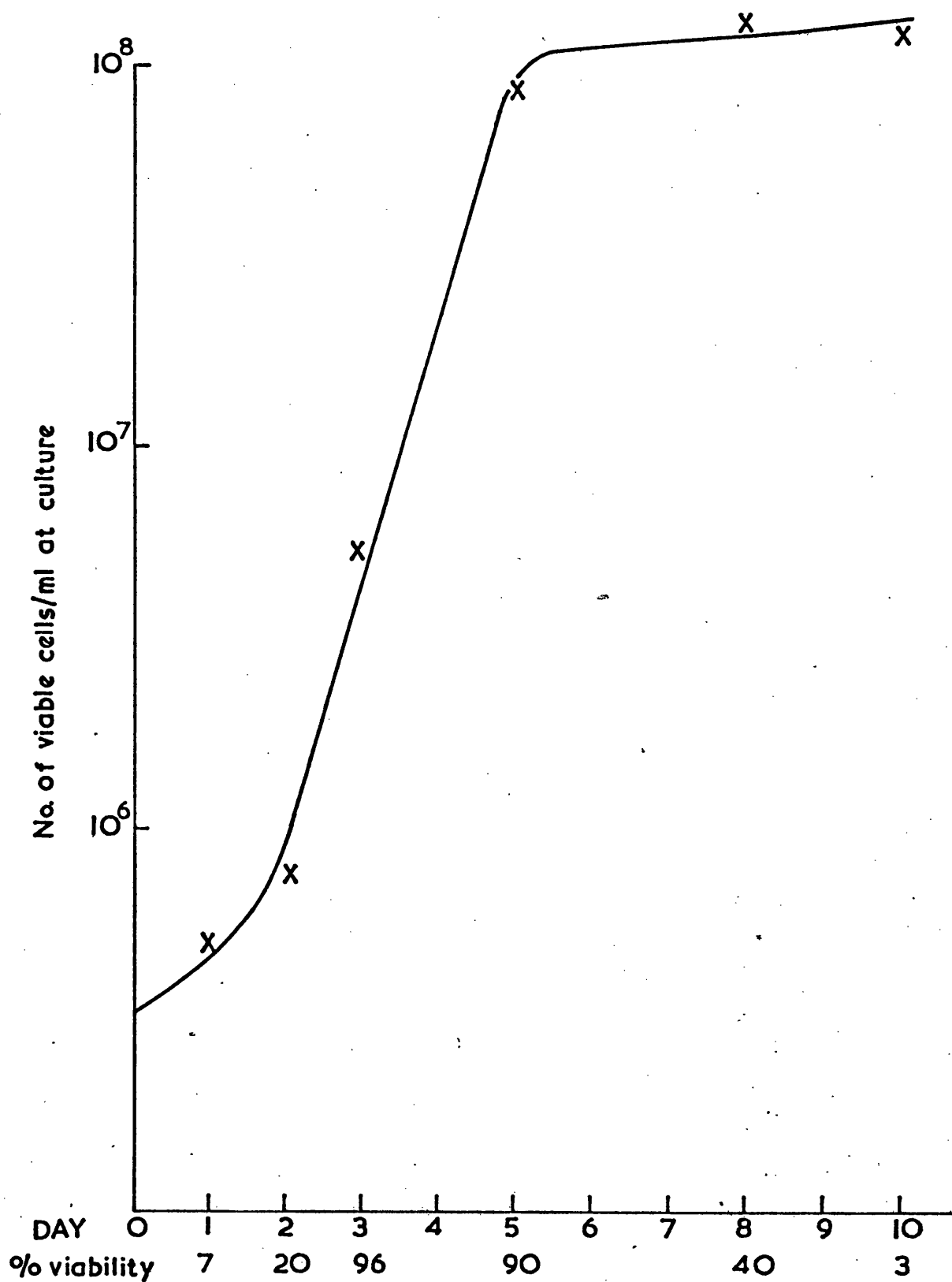


TABLE 5Effect of changing media on % viability of T.ferro oxidans Tf3

<u>liquid medium</u>	to <u>solid medium</u>	<u>% viability</u>
F	F	95.0
F	ST	0.05
θ	ST	14.0
ST	ST	71.0
ST	F	0.01
S	<del>ST</del> F	0.5

Chambers were counted after 48 hours incubation at 30°C

F = ferrous sulphate medium

ST = thiosulphate medium

S = sulphur medium

Effect of atmospheres of argon and hydrogen on growth

To determine if it was an excess of air which inhibited growth of T.ferro oxidans on agarose or if it was an actual requirement for nitrogen that stimulated growth, viable counts and slide chambers were set up under argon + 5% CO<sub>2</sub> and the growth compared with that obtained by incubation under nitrogen. No difference in viability was found and it was concluded that

air was inhibitory. If hydrogen is used to replace nitrogen, growth on plates was unaffected. This does not mean that I.ferro oxidans is able to grow under anaerobic conditions, for this to be the case all oxygen dissolved in the medium must be removed which in practice proved very difficult. Gas analysis of the  $N_2 + 5\% CO_2$  showed it to be oxygen-free. The oxygen requirement for growing cells on 'F' agarose must be very small and able to be satisfied by that dissolved in the medium.

#### Effect of oxygen tension on bacteria growing on plates

An experiment was carried out to determine if exposure to air during incubation inhibited growth but was non-lethal to the cells or if it caused permanent irreparable damage. Strains used in this experiment were Tf3, Tf26 and Tf47. 'F' agarose medium plates were inoculated from a log. phase culture of each by the overlay method and a duplicate set of dilutions incubated in each of 8 anaerobic jars. Four jars were gassed with air + 5%  $CO_2$  and 4 jars with  $N_2 + 5\% CO_2$ . All jars were incubated throughout the experiment at  $30^\circ C$ . After one day's incubation one jar from the air series had the atmosphere changed by evacuation and gassing to  $N_2 + 5\% CO_2$  and similarly one jar from the  $N_2$  series was changed to air. This procedure was repeated on day 3 and day 7. After a total of 11 days incubation all the jars were opened and the number of colonies which had grown were counted. The plates were incubated for a further 20 days and the colonies counted again. The result is given in Table 6 and clearly showed the importance of the atmospheric conditions experienced during the initial 1-3 days. It showed that although growth would not be initiated in air, once growth had been initiated even to the microcolony stage it would continue in air and colonies formed. The experiment also showed that exposure to air was lethal to

TABLE 6

Effect of incubation of air and/or nitrogen of colony  
formation of *T.ferro oxidans* on ferrous sulphate agarose medium

<u>Strain</u>	<u>Number of day of initial incubation</u>		<u>No.of colonies after 11 days</u>
	<u>in air + 5%CO<sub>2</sub></u>	<u>in nitrogen + 5%CO<sub>2</sub></u>	<u>per ml</u>
Tf3	1		$1 \times 10^6$
	3		0
	7		0
	11	0	0
		1	$2 \times 10^6$
		3	$3 \times 10^7$
		7	$3.5 \times 10^7$
	0	11	$3.5 \times 10^7$
Tf26	1		$2 \times 10^6$
	3		$6 \times 10^3$
	7		0
	11	0	0
		1	$1.1 \times 10^6$
		3	$2 \times 10^7$
		7	$2 \times 10^7$
	0	11	$2 \times 10^7$
Tf47	1		0
	3		0
	7		0
	11	0	0
		1	0
		3	$1 \times 10^6$
		7	$2 \times 10^6$
	0	11	$2 \times 10^6$

Incubated at 30°C



T.ferro oxidans for bacteria exposed on plates for a minimum period of time were unable to grow even if the air in the atmosphere was replaced by nitrogen. Some strain variation was found. Tf26 was able to grow and form colonies after 3 days in air.

From slide cultures we have evidence that 2-3 days is the time required for the growth of microcolonies of 'F' agarose and a simple explanation of the results in Table 6 may be that a single cell of T.ferro oxidans exposed on the surface of a plate is sensitive to oxygen but once divisions have taken place and a microcolony formed, the outer cells protect the inner cells and growth continues in the environment controlled by the colony and not the atmosphere around it. However if a single cell is sensitive to oxygen when on an 'F' agarose plate why is it not sensitive when on an ST agarose plate?

A reaction which occurs in 'F' agarose medium, but not ST agarose medium, is the atmospheric oxidation of the ferrous sulphate which is provided as the sole source of energy for the bacteria. When an 'F' agarose plate is incubated in air the ferrous sulphate is oxidised to ferric sulphate which is precipitated and can be seen microscopically in the medium, as is illustrated in Plate 9, and gives the medium a brown coloration. If we consider the surface of an  $\text{FeSO}_4$  plate there is a large fixed area exposed for oxidation. Ferric sulphate is readily formed which may mechanically interfere with cell division as ice crystals do. A more likely explanation is that during the first 24 hours the surface ferrous sulphate is oxidised to ferric sulphate and the bacterial cells after growth initiation are very soon unable to continue due to

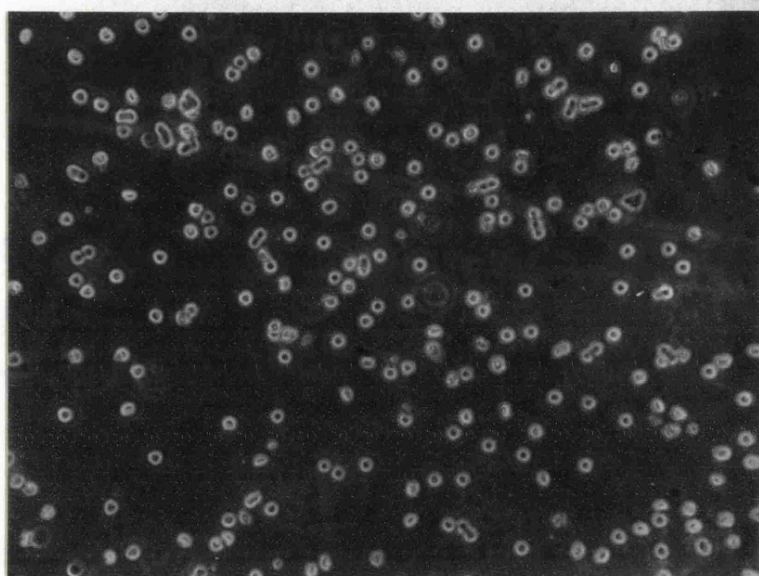


PLATE 9

Precipitate of basic ferric sulphate formed in  
'F' agarose medium.

magnification x 250

starvation of its source of energy. In the absence of atmospheric oxygen the cells have an ample energy supply and are able to obtain what oxygen they require for growth from that dissolved in the medium. The physiological condition of the starved cell renders it sensitive to oxygen or acid pH or some such factor whereas the resting, or growing, cells are not. Tf26 has a tendency for prolonged lag phases, (as will be seen in the next section) and it could be the resting cell which survived the 3 days incubation in air.

T. ferro oxidans was reported to grow and form colonies when plated on a membrane filter supported on Japanese agar and a suitable ferrous-sulphate salts medium (Tuovinen and Kelly 1973). The plates were incubated in air for 15 days, after which time small brown colonies could be seen. The colonies remained small and counting could be assisted by staining the membrane (Tuovinen, personal communication). Using this technique and incubating the membranes on the agar under  $N_2 + 5\%CO_2$ , large colonies clearly visible to the naked eye could be seen 10 days as shown in Plate 10. The top two plates in the photograph show the growth from 10 mls of a culture filtrate and 1 ml of the same culture when incubated in nitrogen. The lower plate is the filter of a 10 ml sample of the same culture incubated in air.

If the atmospheric oxidation of the surface ferrous sulphate is the cause of growth inhibition on agar or agarose, a membrane filter on the surface would greatly retard surface oxidation as a wet membrane only permits gaseous diffusion in solution. Atmospheric oxidation would be retarded and bacterial growth limited by the rate of diffusion of essential nutrients but not starved completely of their energy source.

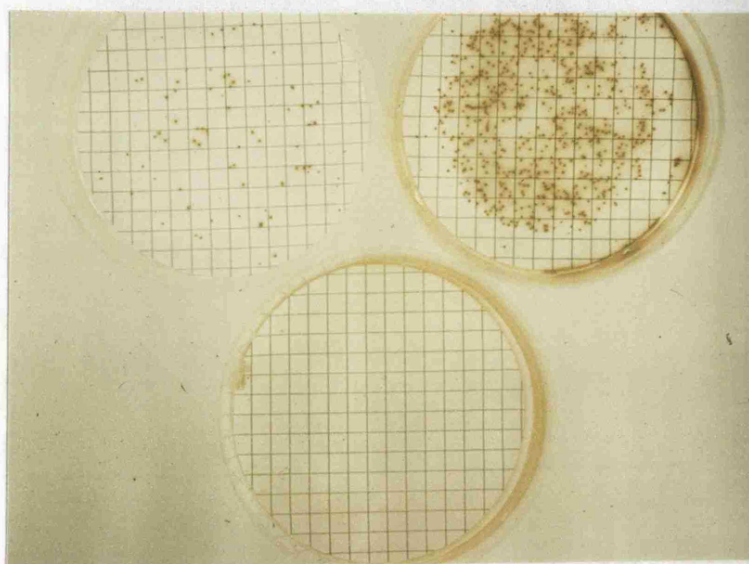


PLATE 10

T.ferro oxidans colonies growing on membrane filters  
incubated at 30°C under N<sub>2</sub> + 5% CO<sub>2</sub> and air + 5% CO<sub>2</sub>  
for 10 days.

0.725 x actual size

### Optimal $pO_2$ for growth

An experiment was carried out to determine the optimal  $pO_2$  for growth of T.ferro oxidans on an 'F' agarose medium. Log: phase liquid cultures of Tf3, Tf18, Tf26 and Tf52 were each suitably diluted and plated by the overlay technique. The plates were incubated in anaerobic jars and evacuated and gassed (x2) with various gas mixtures. The plates were incubated for 10-15 days before the colonies were counted. The gas mixtures used are given in Table 7 and the result shown in Fig. 3. Strain Tf26 shows slight variations but all strains gave the highest viable counts in the atmosphere of minimum oxygen.

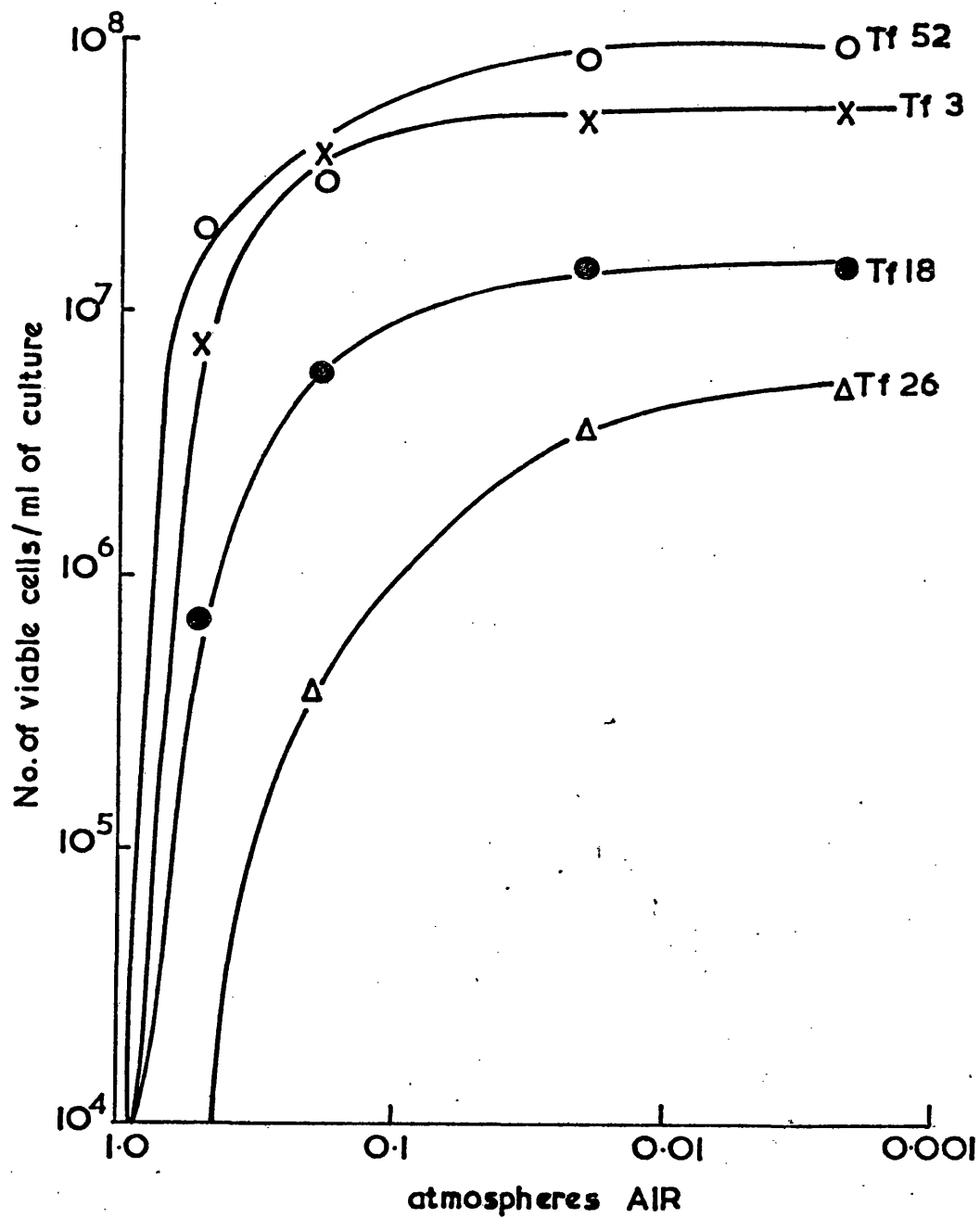
TABLE 7

Gas mixtures used to study the effect of  $pO_2$  on the growth of T.ferro oxidans as estimated by a viable count on ferrous-sulphate agarose medium.

% air(+ 5%CO <sub>2</sub> )	% N <sub>2</sub> (+ 5%CO <sub>2</sub> )	atmospheres AIR	$pO_2$
100	0	0.95	0.225
50	50	0.475	0.1125
30	80	0.195	0.045
2	98	0.0195	0.0045
0	100	less than 0.002	-

FIG.3.

Colony forming ability of T. ferrooxidans strains Tf 3, Tf 18, Tf 26 & Tf 52 under various atmospheric conditions



### Adsorption of *T.ferro oxidans* to solid materials

*T.ferro oxidans* adheres to surfaces which may be the side of a glass culture vessel or the precipitate formed during growth in ferrous sulphate medium. This tendency of these bacteria could be of evolutionary significance for, although their natural liquid environment contains some ferrous sulphate, much of their energy comes from pyrite an insoluble particulate material which may be widely distributed in a rock formation. It would be highly advantageous for the bacteria to be able to attach themselves to their source of energy.

Using a viable count to determine the number of bacteria in suspension in a culture the degree of adsorption could be demonstrated. To a 50ml flask containing 9ml of 'F' salts and either 0.1 gm or 0.5 gm of sterile glass beads, 0.1mm diameter or 0.3mm diameter or sterile pyrite ground to 300 mesh was added 1 ml of a log phase ferrous sulphate grown culture of *T.ferro oxidans* Tf3. A control flask contained only 9ml 'F' salts and the inoculum. The flasks were gently shaken for 5 minutes and left stationary for 1 hour. An 0.2 ml sample was taken from each flask and after suitable dilutions plated on 'F' agarose medium. The flasks were then gently shaken for 3 hours and a second sample taken, allowing the pyrite to settle before sampling. These were also plated on 'F' agarose. All the plates were incubated in anaerobic jars under an atmosphere of  $N_2 + 5\% CO_2$  at  $30^\circ C$  for 10 days when the colonies which had grown were counted. The results are given in Table 8 and show that Tf3 is adsorbed to both glass beads and pyrite with a much greater affinity for the pyrite. This may be related to the increased available surface



TABLE 8

Adsorption of T.ferro oxidans Tf3 to various particulate substances as shown by numbers of bacteria remaining in suspension after 1 hour and 3 hours incubation in contact at 30°C.

Time (hours)	glass beads				pyrite		Control
	0.1mm 1%                  5%		0.3mm 1%                  5%		300 mesh 1%                  5%		
1	1.9x10 <sup>6</sup>	1.7x10 <sup>6</sup>	5.7x10 <sup>5</sup>	3x10 <sup>5</sup>	3x10 <sup>4</sup>	5x10 <sup>3</sup>	1.2x10 <sup>7</sup>
3	1.3x10 <sup>5</sup>	3x10 <sup>5</sup>	7x10 <sup>5</sup>	5x10 <sup>5</sup>	1.5x10 <sup>3</sup>	1.3x10 <sup>3</sup>	1.4x10 <sup>7</sup>

bacterial counts given 1 ml of culture.

area but little difference was found in bacterial adsorption between 1% and 5% glass beads. Vigorous shaking did not remove the bacteria from the pyrite. Slide-culture chambers were used to determine the viability of those bacteria remaining in suspension and whereas the control culture had a 79% viability on the slide cultures, the bacteria in suspension above the 5% pyrite had only a 16% viability indicating that viable bacteria are preferentially adsorbed on to pyrite.

#### Summary of growth on a solid medium

A method of growing T.ferro oxidans on a solid medium was found. The atmosphere in which the bacteria were incubated was more important than the agent used to solidify the medium. Only by reducing the oxygen in the atmosphere during the first 3 days after inoculation on to plates could colony formation be initiated. This was achieved in anaerobic jars by replacing the air with nitrogen + 5% carbon dioxide. The 5% CO<sub>2</sub> was never shown to be necessary but was always added.

Growth was estimated by a viable count. Behaviour of the bacteria was observed microscopically using slide-culture chambers and it was found that the 5.5cm petri dishes could be used in a similar manner for microscopic examination if the plates contained no more than 3ml of media.

Growth experiments on 'F' agarose showed that many cultures of T.ferro oxidans were mixed population of autotrophs as illustrated in Plate 11. This is a photograph of Tf26 as received from the Chamber of Mines in South Africa and believed to be a pure culture as it had been kept for years in liquid ferrous sulphate medium.

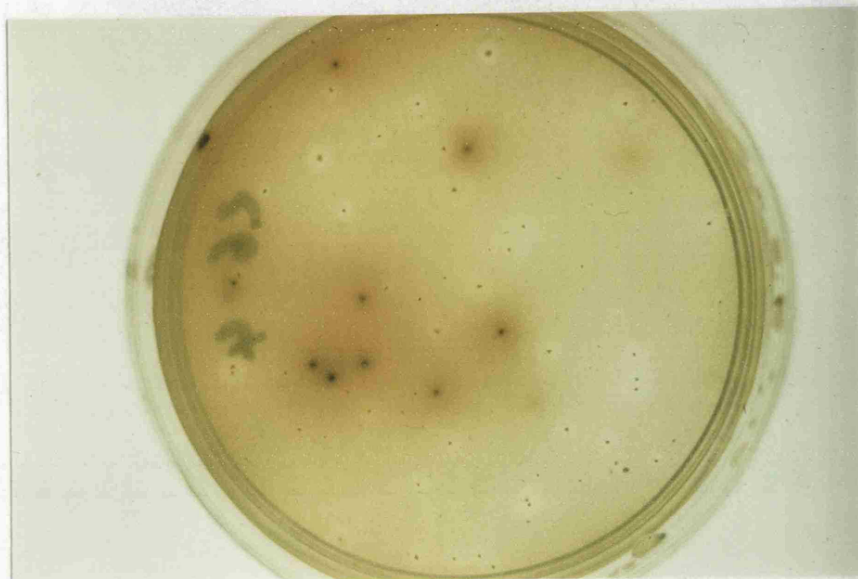


PLATE 11

Mixed culture of Thiobacilli growing on 'F' agarose  
incubated at 30°C under N<sub>2</sub> + 6% CO<sub>2</sub> for 10 days

Actual size

As can be seen it is a mixed culture of I.ferro oxidans which are deep brown colonies surrounded by a precipitate and a 'non-iron-precipitating' (NIP) bacterium which formed cream coloured colonies and were surrounded by a clear zone. (This may be I.acidophilus which is able to utilise the galactose in the agarose for its carbon source).

The development of a method for purification of I.ferro oxidans has shown that a pure culture of I.ferro oxidans is non-motile irrespective of the growth medium. I.thio oxidans is very motile as a young culture in sulphur medium and is motile in thiosulphate and tetrathionate media. The facultative autotrophs are all reported as being motile. If motile organisms are seen in a liquid ferrous sulphate medium the culture is a mixed population which can be separated if the correct solid media are used.

Using 'F' agarose medium all strains of I.ferro oxidans were purified before use in most experimental work. Stringent care was taken with Tf3 for the nitrogen fixation experiments which will be discussed in that section.

The ability of I.ferro oxidans to adsorb to pyrite and other surfaces was noted and must be remembered when growth on pyrite, as energy source, is being determined for the number of bacteria in suspension is only a small percentage of those present in the culture.

### SECTION III

#### Growth of *T. ferro oxidans* in liquid media

Experiments with *T.ferro oxidans* growing in flasks in a liquid chemically-defined ferrous sulphate medium were carried out to determine the optimal growth conditions in relation to the temperature, pH and chemical composition of the medium and to identify any strain variations.

A selection of strains was used and as with the growth on solid media, strain Tf3 was used throughout the work as a reference strain in that the behaviour of all other strains was compared to it.

#### Ferrous sulphate media for growth of *T.ferro oxidans*

There are two media commonly quoted for growth of *T.ferro oxidans* but they are in fact concentration variations of the same basal salts. They are quoted extensively as 'F' medium of Leathen, Kinsel and Braley (1956) and '9K' medium of Silverman and Lundgren (1959). The comparative composition of the two media are given in Table 9. The '9K' refers to the fact that the concentration of iron is 9000 parts per million (ppm). In 'F' medium the iron concentration is only 200 ppm.

A standard procedure for experiments in flask culture was adopted. A 500 ml flask containing 100 ml of test medium was inoculated and weighed. For each assay a standard sample volume of 2 ml was taken. On each day of assay each flask was weighed and any weight loss due to evaporation was calculated as the original weight minus the weight of previous samples taken, was

made up with sterile distilled water before any further samples were taken. Growth in liquid medium was estimated by the disappearance of ferrous sulphate from the medium and the appearance of ferric sulphate by the colorimetric determination of  $\text{Fe}^{3+}$  as the yellow  $\text{FeCl}_3\text{-HCl}$  complex (Schnaitman, Korczynski and Lundgren 1969). Full details of this assay are given in 'Methods and Materials'.

TABLE 9

Comparison of media for growth of Thiobacillus ferro oxidans

<u>Components</u>	'F'	'9K'
Basal salts	9	9
$(\text{NH}_4)_2 \text{SO}_4$	0.15	3.0
	0.05	0.10
$\text{K}_2\text{HPO}_4$	0.05	0.50
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.50	0.50
$\text{Ca}(\text{NO}_3)_2$	0.01	0.01
$\text{H}_2\text{O}$	1000 ml	700 ml
10N $\text{H}_2\text{SO}_4$	-	1.0 ml
Energy source		
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	10ml(of 10% w/v)	300ml(of 14.74% w/v)
pH	3.5	3.2 - 3.6

### Comparison of growth on two ferrous sulphate media

The first experiment to compare growth in 'F' and '9K' media showed a marked difference between the two media (Fig.4). In the 'F' medium all the iron was used up by day 3. The ferrous sulphate concentration in the 'F' medium was increased to 25 mg/ml to provide more energy source so that a comparison of the basal salts could be made. Fig. 5 shows two strains, Tf3 and Tf18 growing in '9K' medium, which contains 45 mg/ml  $\text{Fe SO}_4$ , and the new 'F' medium with 25 mg/ml  $\text{Fe SO}_4$ . Both media support growth equally well. Growth in the new 'F' medium only begins to slow down when the supply of energy becomes exhausted.

By growing *T.ferro oxidans* strain Tf3 and Tf18 in 'F' and '9K' basal salts with concentration of  $\text{Fe SO}_4$  at 45 mg/ml and 25 mg/ml it was shown that growth was related to the final concentration of  $\text{Fe SO}_4$  (Fig.6) and the composition of the basal salts had little effect on the rate of ferrous sulphate utilisation by the bacteria.

Growth in '9K' medium is accompanied by the formation of a heavy precipitate. This was noted by Silverman and Lundgren (1959) and ascribed to the formation of ferrous and ferric phosphate. At the pH of the medium, pH 3.0-3.6, basic ferric sulphate will also be precipitated. The precipitate was very difficult to redissolve even in concentrated hydrochloric acid and it interfered with the colorimetric determinations. Silverman and Lundgren (1959) gave no reason for the large increase in  $(\text{NH}_4)_2\text{SO}_4$  concentration or for the increase in phosphate. Phosphate is essential for the oxidation



FIG. 4.

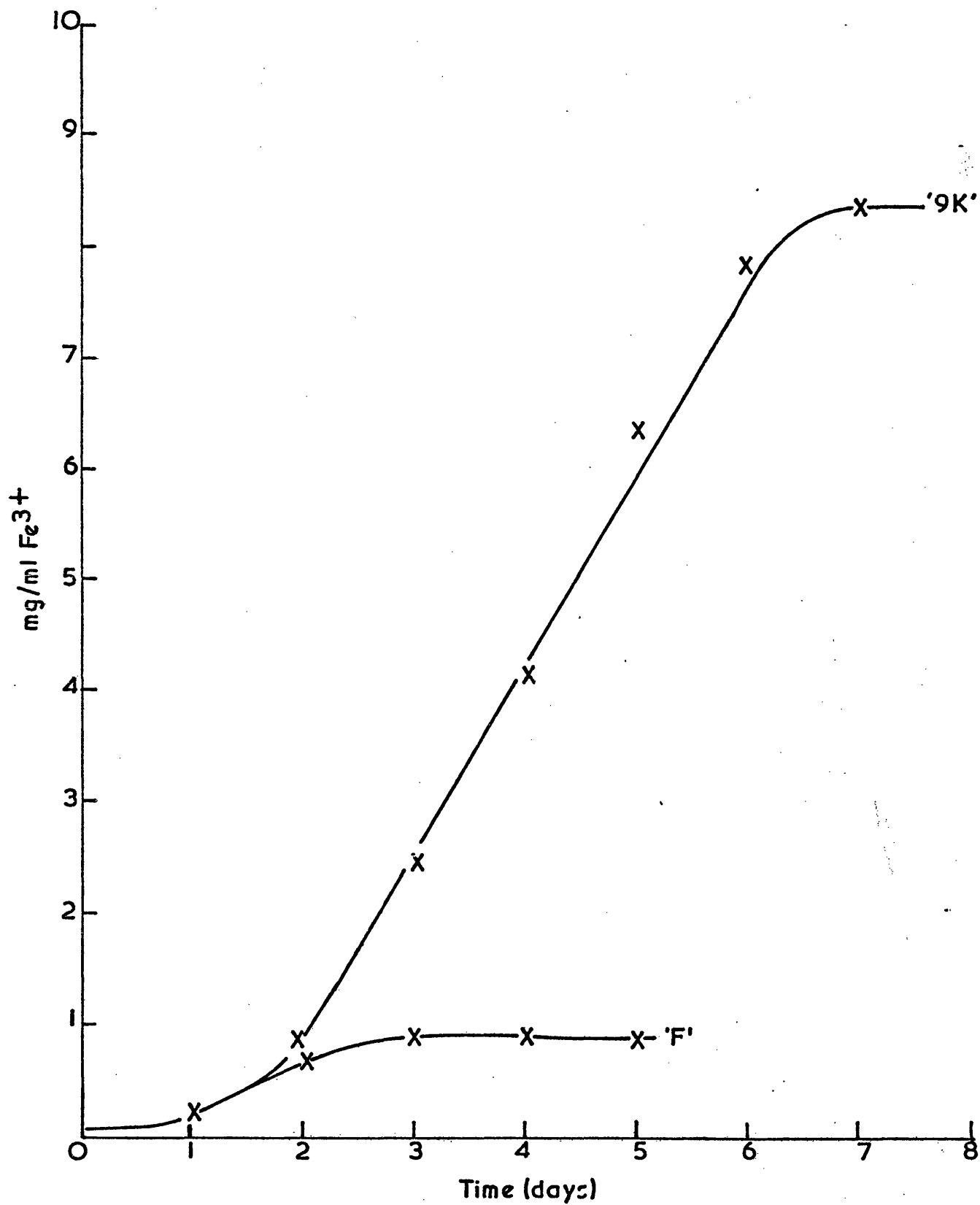
Growth of T.ferrooxidans Tf3 in F & 9K medium at 30°C

FIG.5

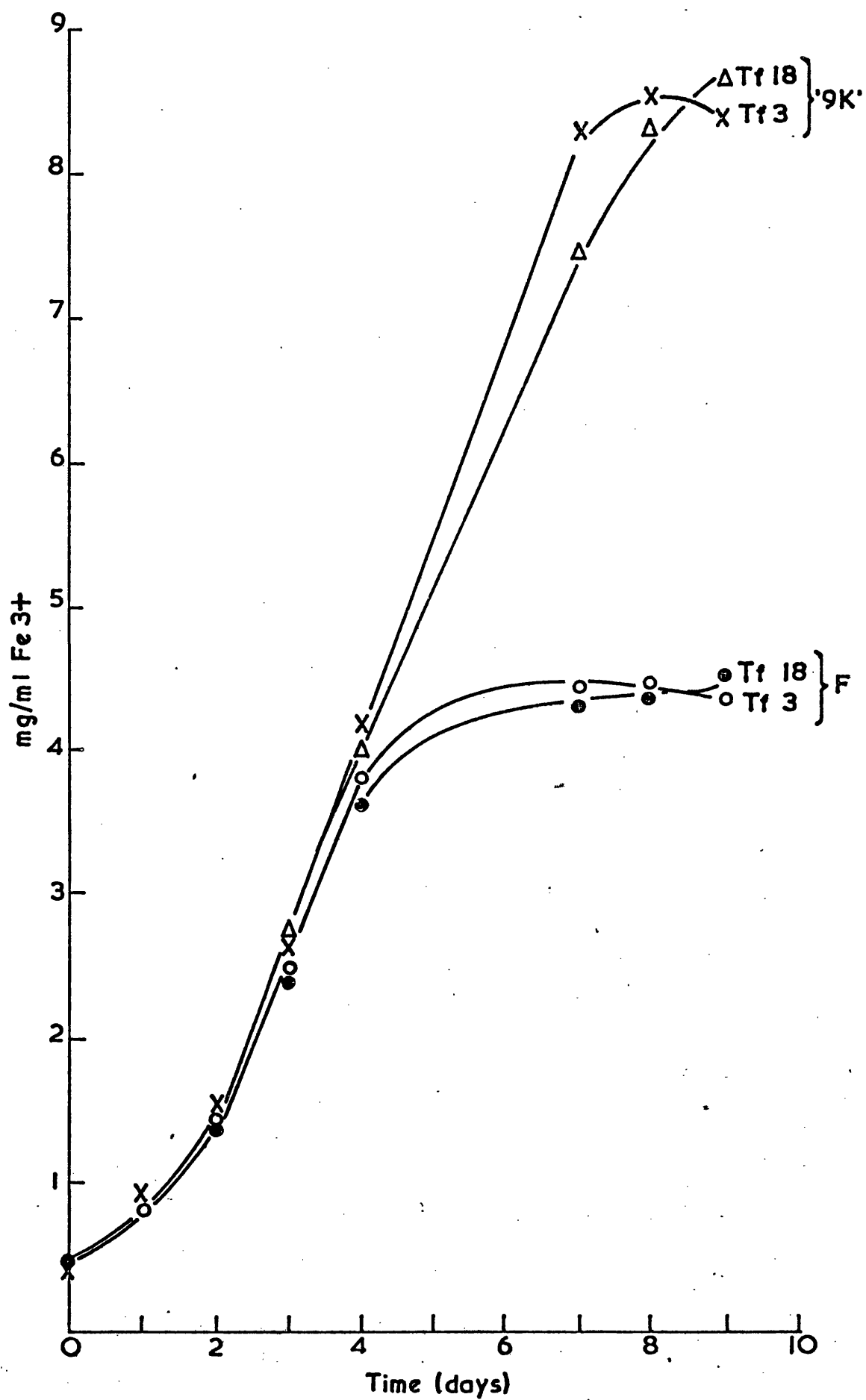
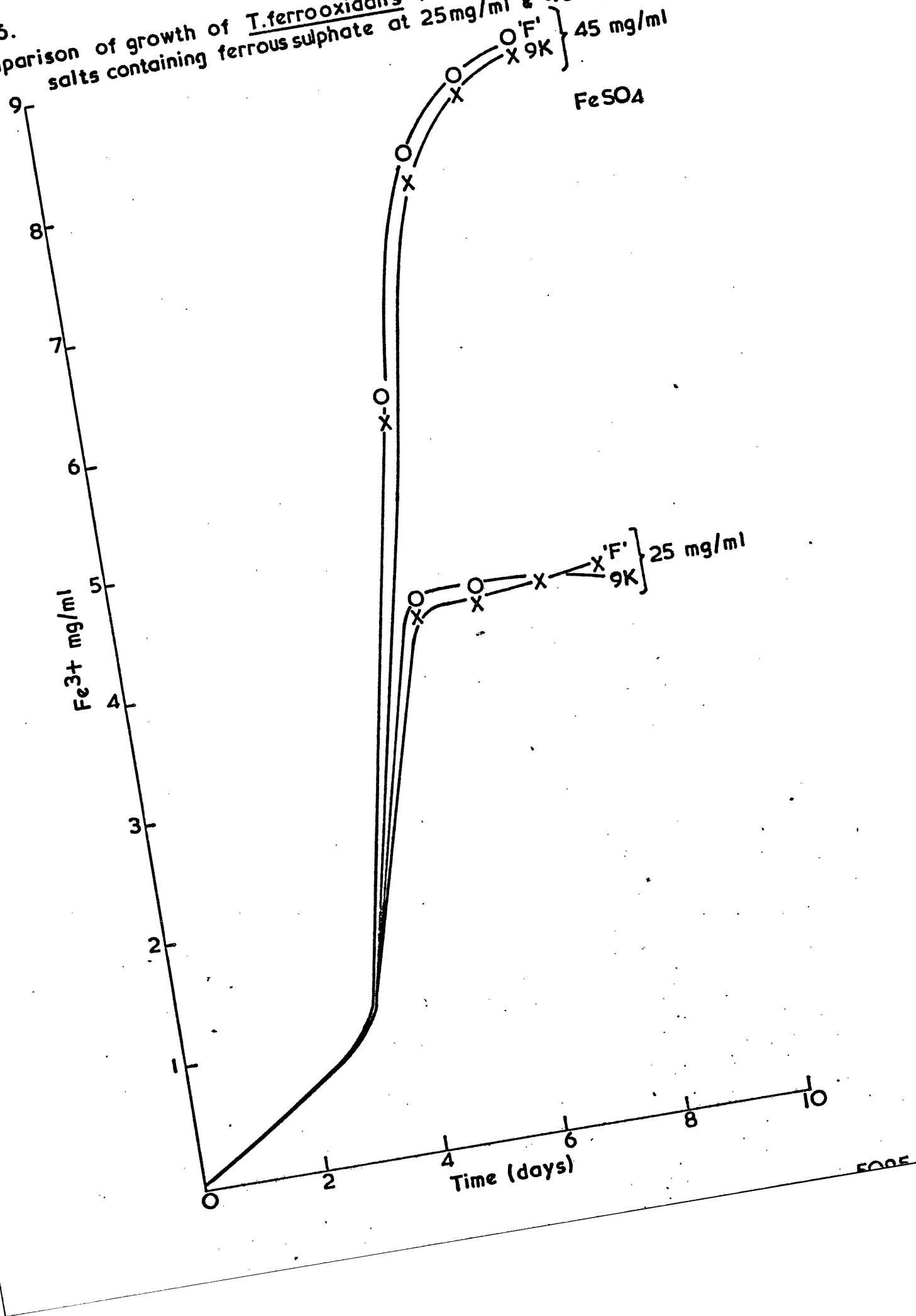
Growth of T.ferro oxidans strains Tf3 & Tf18 on 'F' and '9K' media

FIG.6.  
Comparison of growth of *T.ferrooxidans* Tf 3 on 'F' salts and 9K' salts containing ferrousulphate at 25mg/ml & 45 mg/ml



of ferrous iron (Beck and Shafia 1964) but the phosphate present, both media 'F' and '9K' was far in excess of limiting concentrations. As no advantage in the use of '9K' basal salts was found and the precipitate formed interfered with the assay it was decided to use Leathen and Braley's 'F' salts (1956) with an increased ferrous sulphate concentration of at least 25 mg/ml for further work.

The rate of growth of T.ferro oxidans as estimated by a viable count closely paralleled the rate of ferrous sulphate utilisation as can be seen in Figs. 7 and 8. By using the viable count to estimate cell densities in a liquid ferrous-sulphate culture it was found that the maximum cell density was directly related to the concentration of ferrous sulphate. The basal salts had no effect. It was shown that 45 mg/ml Fe SO<sub>4</sub> supported  $1.5 \times 10^8$  cells/ml and 25 mg/ml Fe SO<sub>4</sub> supported  $8 \times 10^7$  cells/ml. Using these figures it can be estimated that the original 'F' medium, at 1 mg/ml Fe SO<sub>4</sub>, could only support  $3 \times 10^6$  cells/ml. Greater cell densities of T.ferro oxidans could not be achieved by simply increasing the ferrous sulphate concentration. Silverman and Lundgren (1959) showed that 9000 ppm to be the optimum for high cell yields and that a progressive increase in iron concentration failed to increase the final cell yields and progressively decreased the growth rate. Solubilities of CO<sub>2</sub> in water are affected by the 'salting out' effect of small electrolyte ions. (Glasstone 1953) and Tuovinen and Kelly (1973) have proposed that the high levels of ferrous sulphate in the culture media of Silverman and Lundgren (1959) are likely to depress the solubility of CO<sub>2</sub> which would be expressed by limited growth rate and poor bacterial cell yields.

FIG.7.  
Growth of T.ferrooxidans Tf3 as estimated by a  
viable count

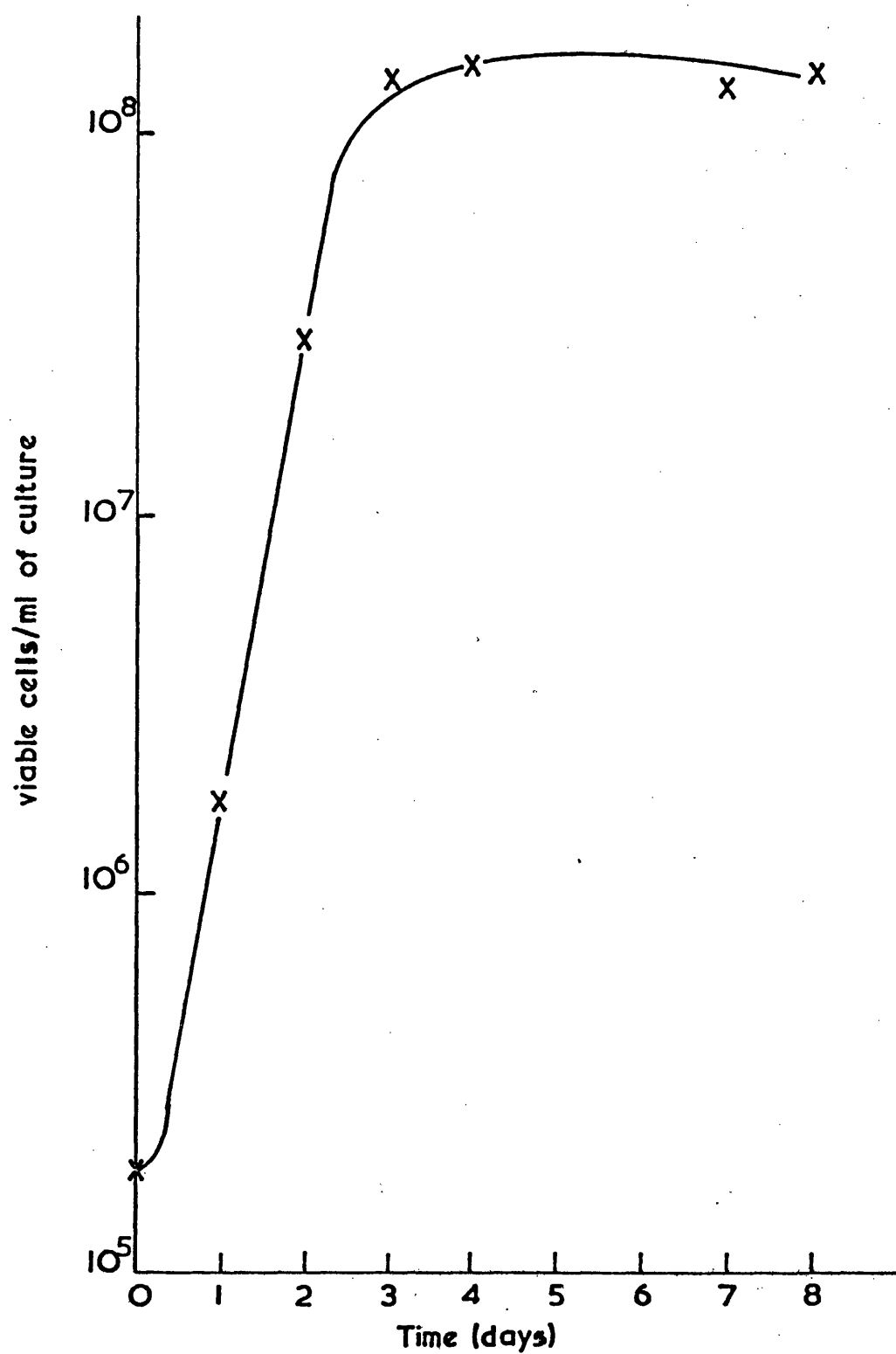
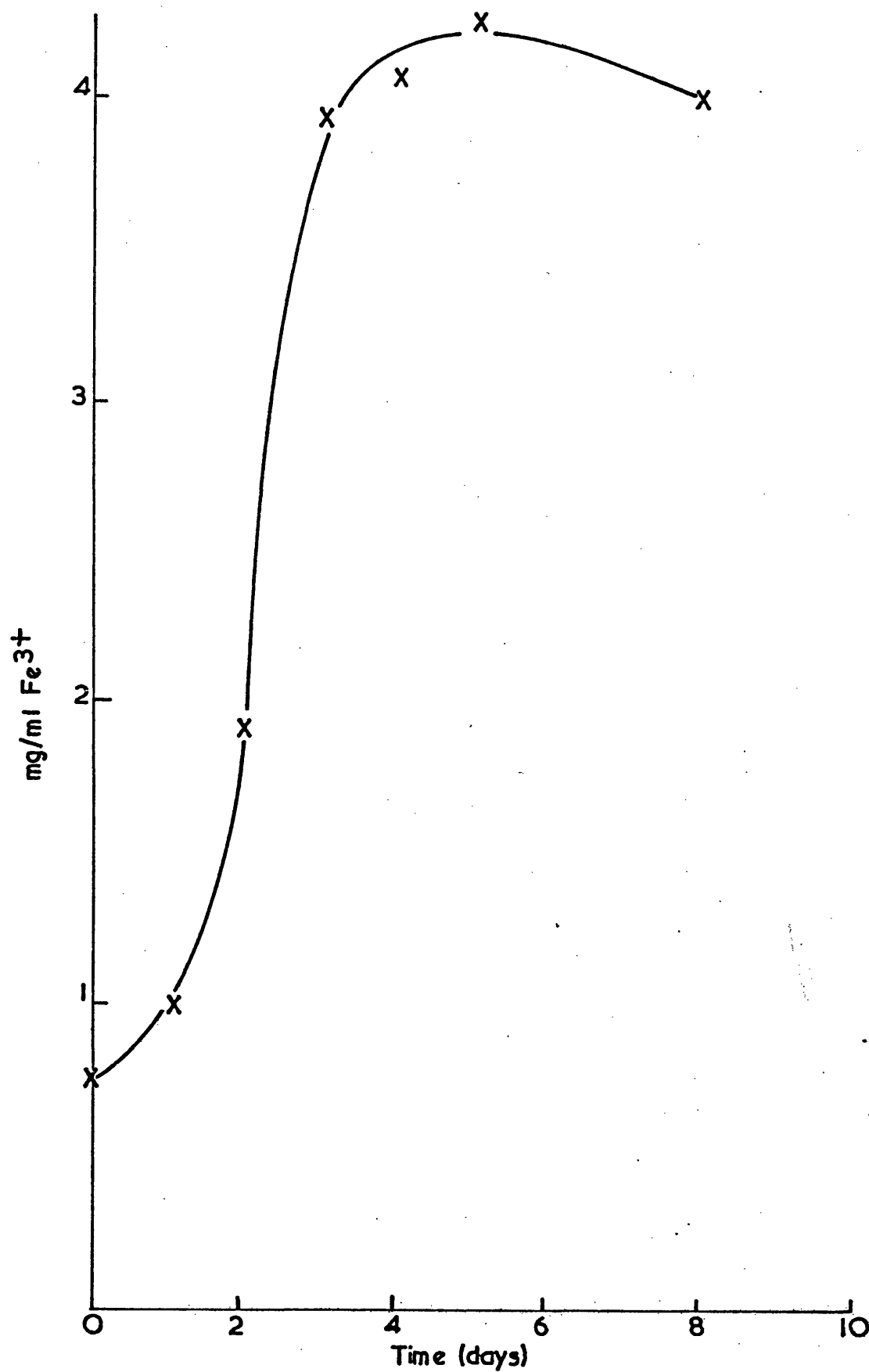


FIG.8

Growth of T. ferrooxidans Tf3 as estimated by the production of ferric sulphate.



Growth requirements by T.ferro oxidans for substances other than ferrous sulphate were looked at and found to be very small. Growth would proceed to a limited extent in tap water and  $\text{Fe SO}_4$ . Fig. 9 shows the growth of Tf3 in three growth limiting media as compared with 'F' salts; all media contained 25 mg/ml  $\text{Fe SO}_4$  and were pH 2.2. The result showed that growth in one tenth the concentration of 'F' salts supported growth until day 5 when growth became limited. Growth in the absence of a soluble inorganic nitrogen source was included as we were interested in the ability of T.ferro oxidans to fix atmospheric nitrogen. The medium had a pH of 2.2 and the atmosphere, in which the culture grew, was rich in ammonia compounds. This graph, therefore, is more representative of the rate of ammonia absorption than nitrogen fixation. The slope of the ' $\text{FeSO}_4$  only' curve suggests that this may also be nitrogen limited.

#### Effect of aeration of the medium on growth

The effect of aerating flask cultures of T.ferro oxidans by incubating on an orbital shaker was studied. Oxygen and carbon dioxide must be taken from the air into solution for utilisation by the bacteria and because of the relative concentration of each in the atmosphere ( $20.95\%(\text{v/v})\text{O}_2$  and  $0.03\%(\text{v/v})\text{CO}_2$ ) there is a greater chance of  $\text{CO}_2$  becoming limited.

A comparison of the effect of incubation of cultures Tf3 and Tf18 in static and aerated (by shaking) environments at  $30^\circ\text{C}$  are shown (Fig.10). Aerating the cultures reduced the period of the lag phase but did not alter the slope of the graph during the logarithmic phase of growth. At temperatures of  $35^\circ\text{C}$  or with vigorous aeration



FIG. 9.

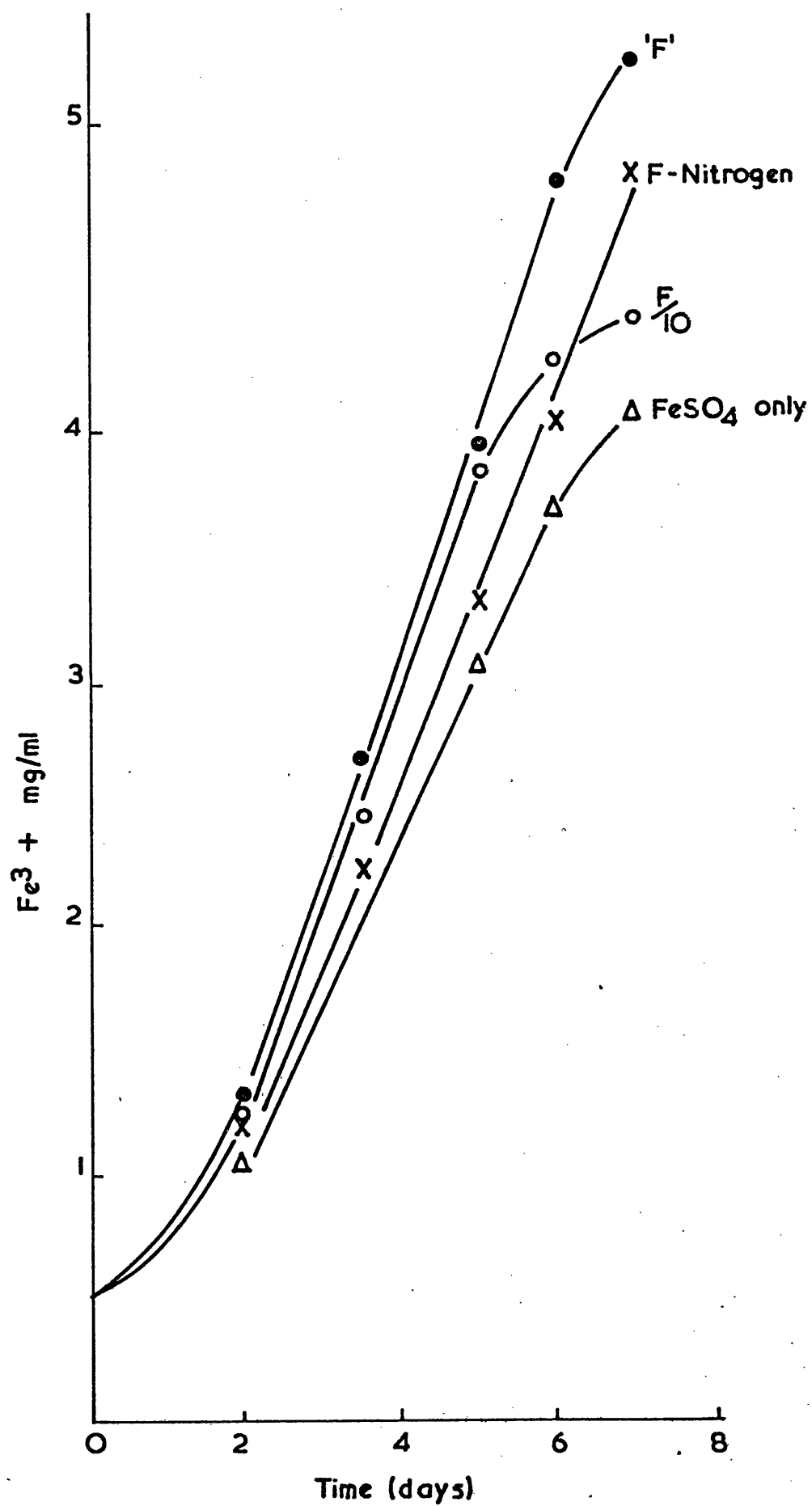
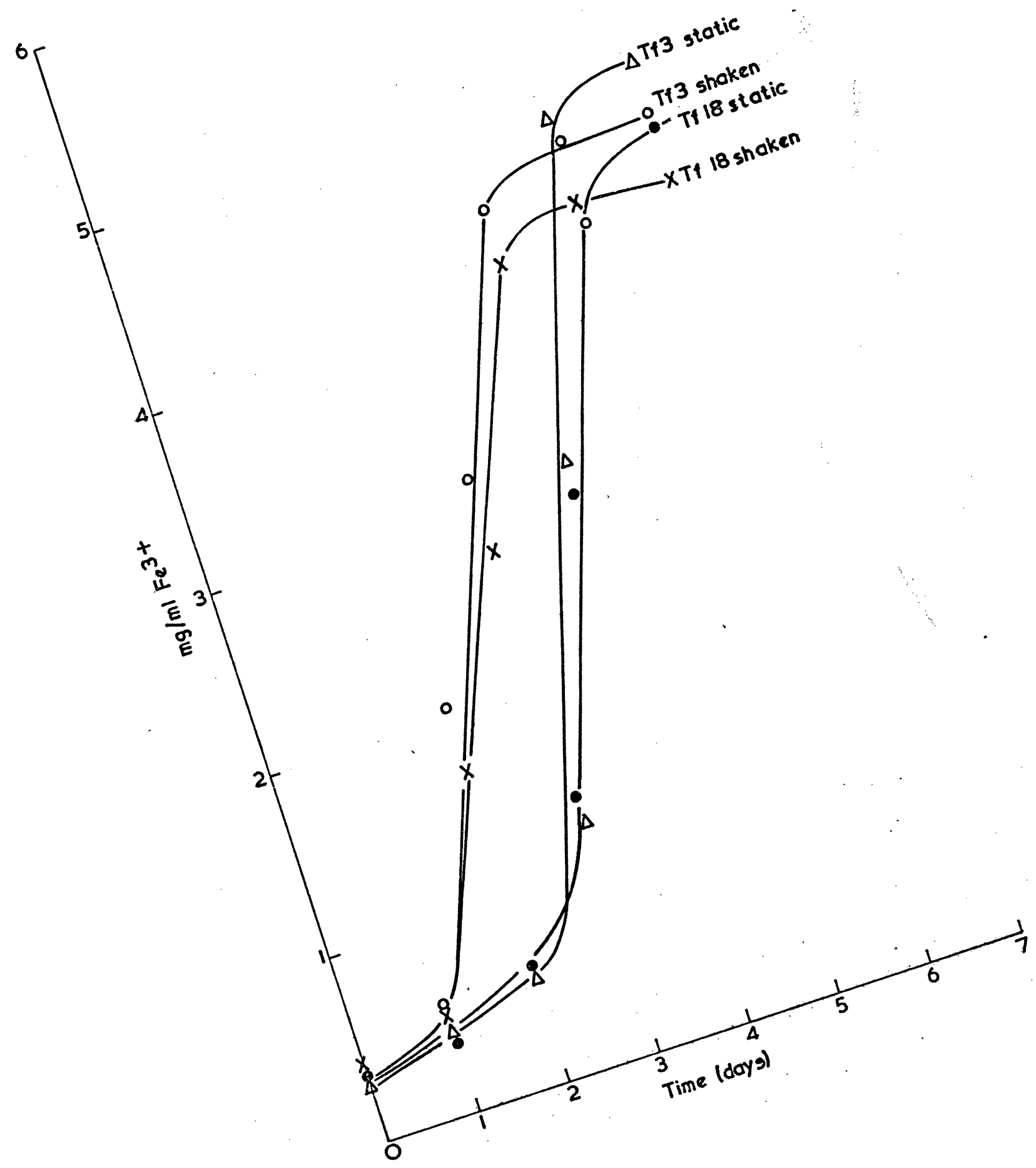
Growth of T.ferrooxidans Tf 3 on various ferrous sulphate media

FIG.10.  
Effect of static and shaken incubation on the growth of T.ferrooxidans  
Tf 3 and Tf18



at 30°C a competition appeared to occur between the bacteria and the oxygen for the available ferrous sulphate. This can be shown by estimating cell numbers by a viable count in conjunction with chemical estimation for ferrous iron utilisation. The result of such an experiment with Tf3 in an aerated culture at 35°C as compared with static cultures at 35°C and 30°C is shown in Figs. 11 and 12. The iron estimation shows a rapid utilisation of ferrous sulphate when the culture is aerated at 35°C. Both static cultures show a longer lag phase but once utilisation of ferrous sulphate occurs it is most rapid at 35°C. If however we look at viable counts taken during this experiment, Fig. 12, we find there was very little difference in the bacterial growth rate but the culture shaken at 35°C was depleted of available ferrous iron by the fourth day and the final cell density was  $3 \times 10^7$  bacteria/ml. In the static cultures growth continued and the final cell density reached at 30°C was  $2 \times 10^8$  bacterial/ml. If the final cell density is used as a rough estimate of ferrous sulphate available to the bacteria the experiment shows that as much as 50% of the ferrous sulphate is oxidised by the atmosphere if cultures are shaken at 35°C.

#### Effect of incubation in $N_2 + 5\% CO_2$ of liquid cultures

In contrast to growth on a solid ferrous sulphate medium no oxygen sensitivity of T. ferro oxidans was found during growth in liquid media and no effect was found by incubating cultures in an atmosphere of  $N_2 + 5\% CO_2$  either to the growth rate or the final cell densities. Oxygen sensitivity during nitrogen fixation by

FIG. 11

Effect of static and shaken incubation on growth of T. ferrooxidans Tf3 as estimated by appearance of ferric sulphate

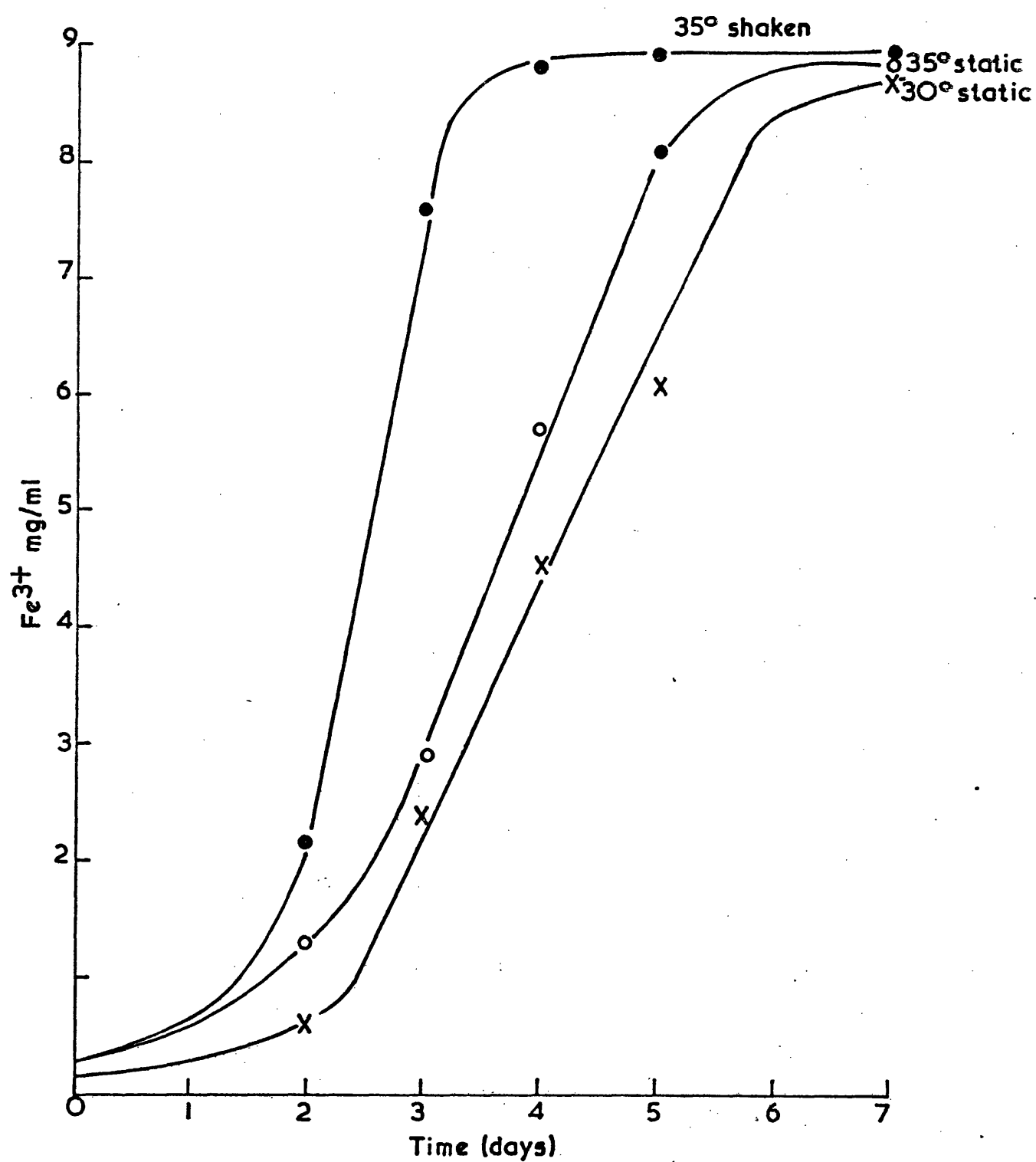
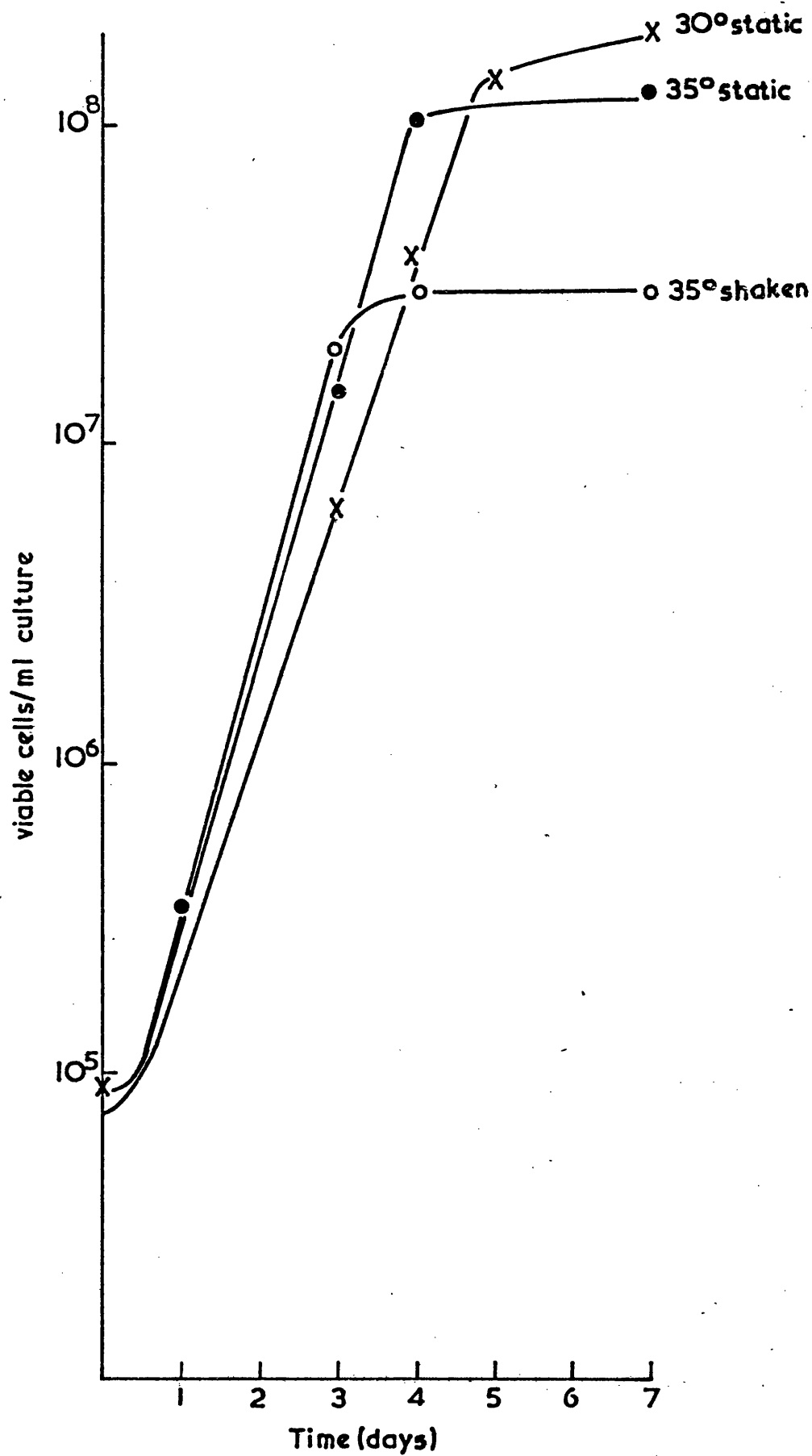


FIG. 12

Effect of static and shaken incubation on growth of T.ferrooxidans Tf3 as estimated by a viable count



T.ferro oxidans was observed and will be discussed later.

#### Effect of temperature on growth

The rates of utilisation of ferrous sulphate by T.ferro oxidans strain Tf3, as an estimate of growth, at various temperatures from 15°C to 35°C are shown, Fig. 13. The medium was 'F' salts with 25 mg/ml Fe SO<sub>4</sub> with static incubation. Growth occurred between 15°C and 35°C and it was interesting to note that at 15°C the lag phase was extended but once growth was initiated it was not of an appreciably lower rate. Growth at 10°C was not detected over a period of 25 days. A working temperature of 30°C was chosen for routine work as atmospheric oxidation of the ferrous sulphate became a problem at 35°C and interfered in long term experiments. No strain variation with respect to temperature was observed in many strains of T.ferro oxidans tested.

#### Effect of pH of medium on growth

The effect of the initial pH of the growth medium was tested over the range pH 1.0–pH 6.0 on the growth of various strains of T.ferro oxidans at 30°C. The pH was not controlled during the experiment and because the medium itself is only poorly buffered the pH of the medium did alter during the experiments. Acid production by bacteria during growth in ferrous sulphate medium is shown in Fig. 14 where T.ferro oxidans strain Tf3 was inoculated into 'F' medium at pH 2.3 and pH 3.2 and incubated at 30°C. Iron estimation and pH reading were taken daily and by day 4 the pH of both cultures was pH 2.3. By day 7, when the bacterial growth was limited by iron depletion, the medium was pH 2.0. Attempts to stabilise the pH by the incorporation of phthalate, citrate and citrate-phosphate buffers were unsuccessful since all the buffers were too toxic at concentrations which had the capacity to buffer (Silverman and Lundgren 1959).

Effect of temperature of incubation on growth of T.ferro oxidans Tf3

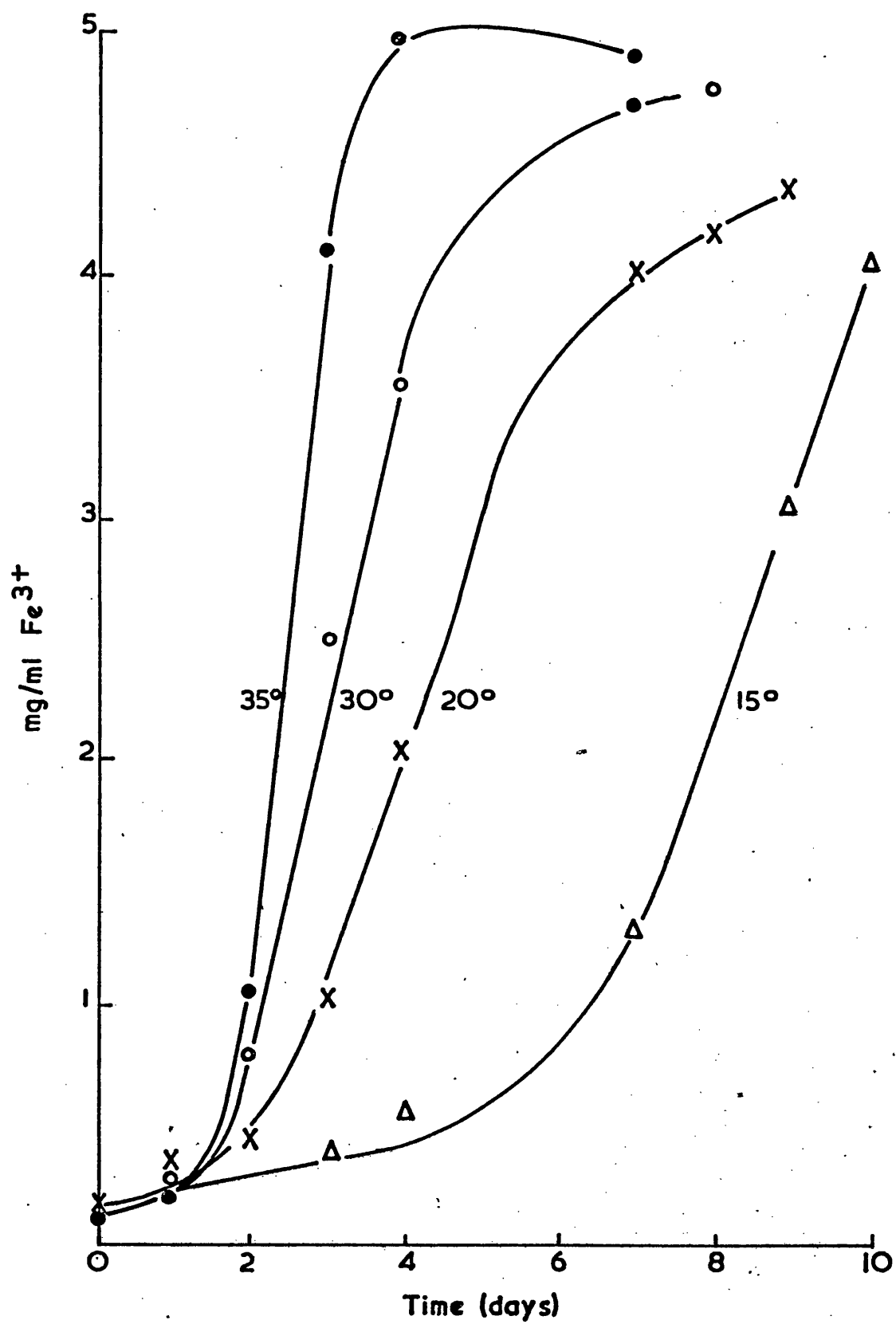
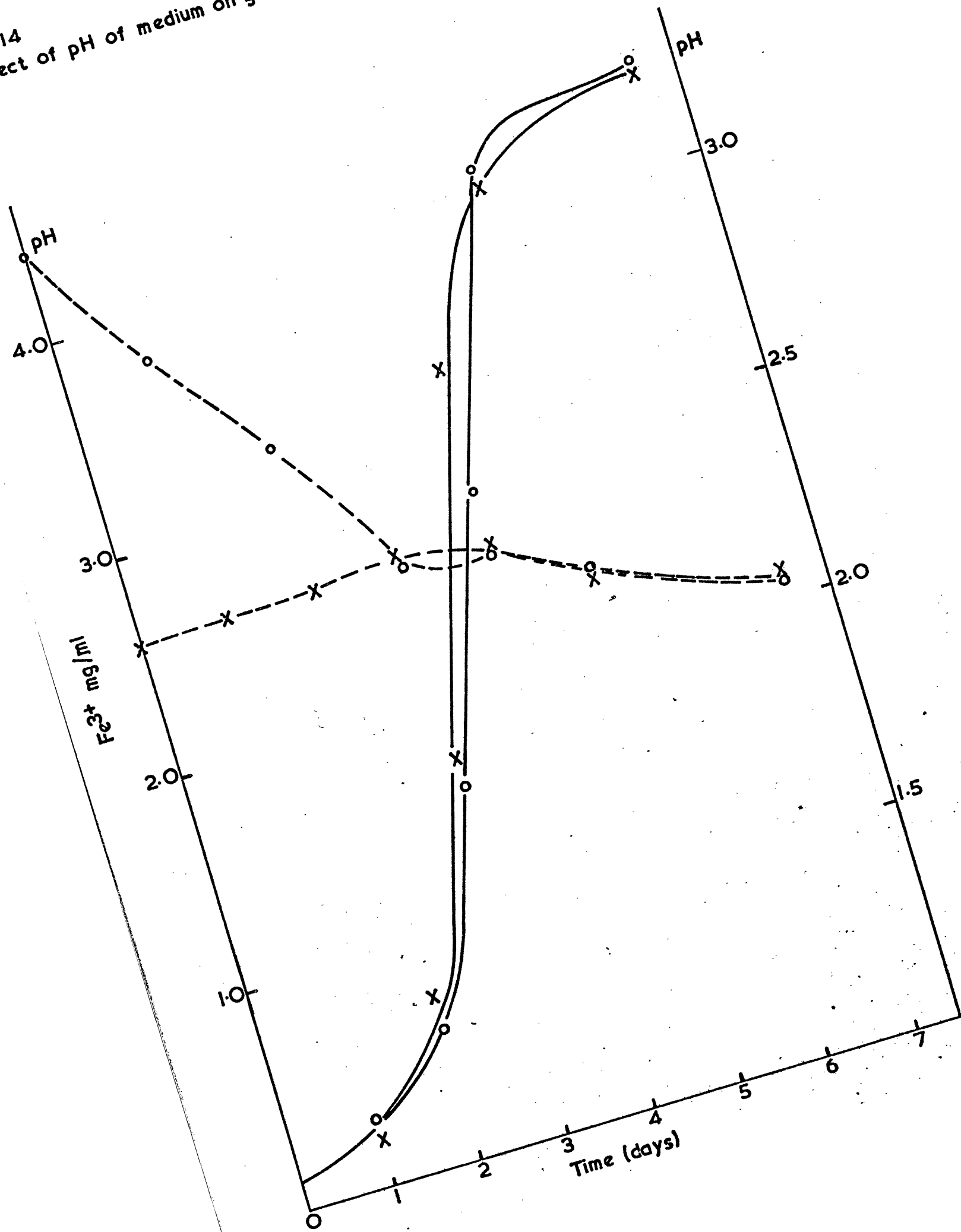


FIG 14  
Effect of pH of medium on growth of T. ferrooxidans T13





Some strain variation was observed in the response of T.ferro oxidans to the initial pH of the growth medium from pH 1.0 - pH 6.0. The most characteristic response is illustrated for strain Tf3 in Fig.15. No growth occurred at pH 1.0 and at pH 4.0 and pH 6.0 an increase in the lag phase was found but the bacterial growth was not greatly affected.

Figure 16 shows the response of Tf18 to the pH of the medium. Unlike any other strain tested Tf18 grew at pH 1.0 after a lag phase of 7-9 days. Growth at pH 1.0 - 6.0 was similar to that of Tf3. Growth at pH 1.0 may have been the result of a spontaneously arising acid tolerant mutant from within the bacterial inoculum or it may be the result of adaptation of the inoculum to grow in the highly acidic condition. Tuovinen and Kelly (1973) were only able to adapt strain Tf3 to grow at pH 1.3 by successive subculture into media of progressively greater acidity. If the 9 days represents the time required for a chemical change in the medium to a more favourable pH for bacterial growth it is surprising that it did not occur in other cultures.

Strain Tf26 showed the greatest sensitivity to the pH of medium for growth initiation (Fig.17) but it was only the lag phase which was affected and once growth got underway it proceeded as with other strains. This South African Chamber of Mines strain appeared to be more sensitive to laboratory conditions than other strains so far tested.

#### Effect of pH and temperature on growth

Experiments with Thiobacillus ferro oxidans Tf3 on the combined effect of pH of medium and temperature of incubation

FIG.15  
Effect of initial pH of medium on growth of *T. ferrooxidans* Tf 3

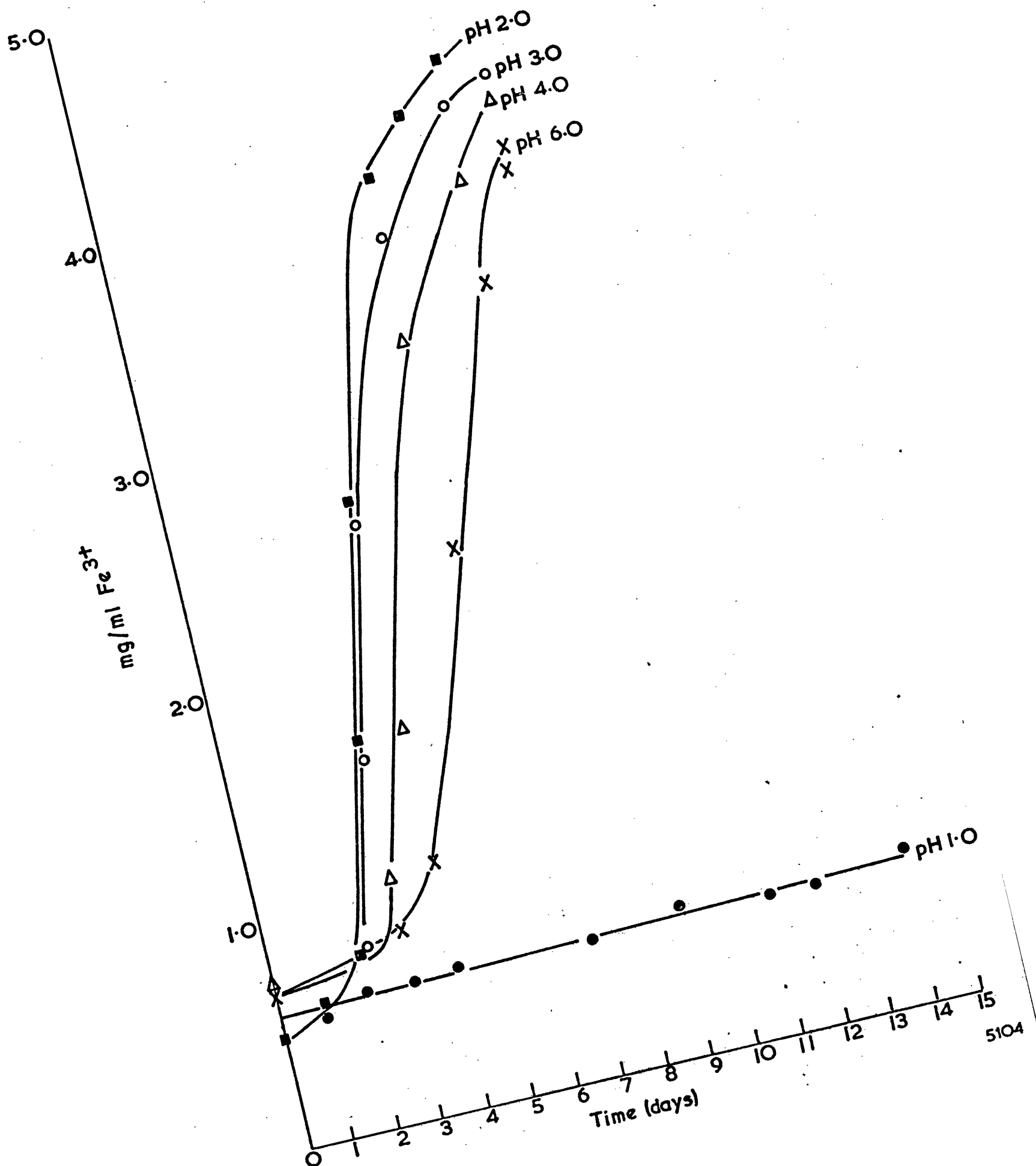


FIG.16  
Effect of initial pH of medium on growth of T. ferrooxidans  
Tf18

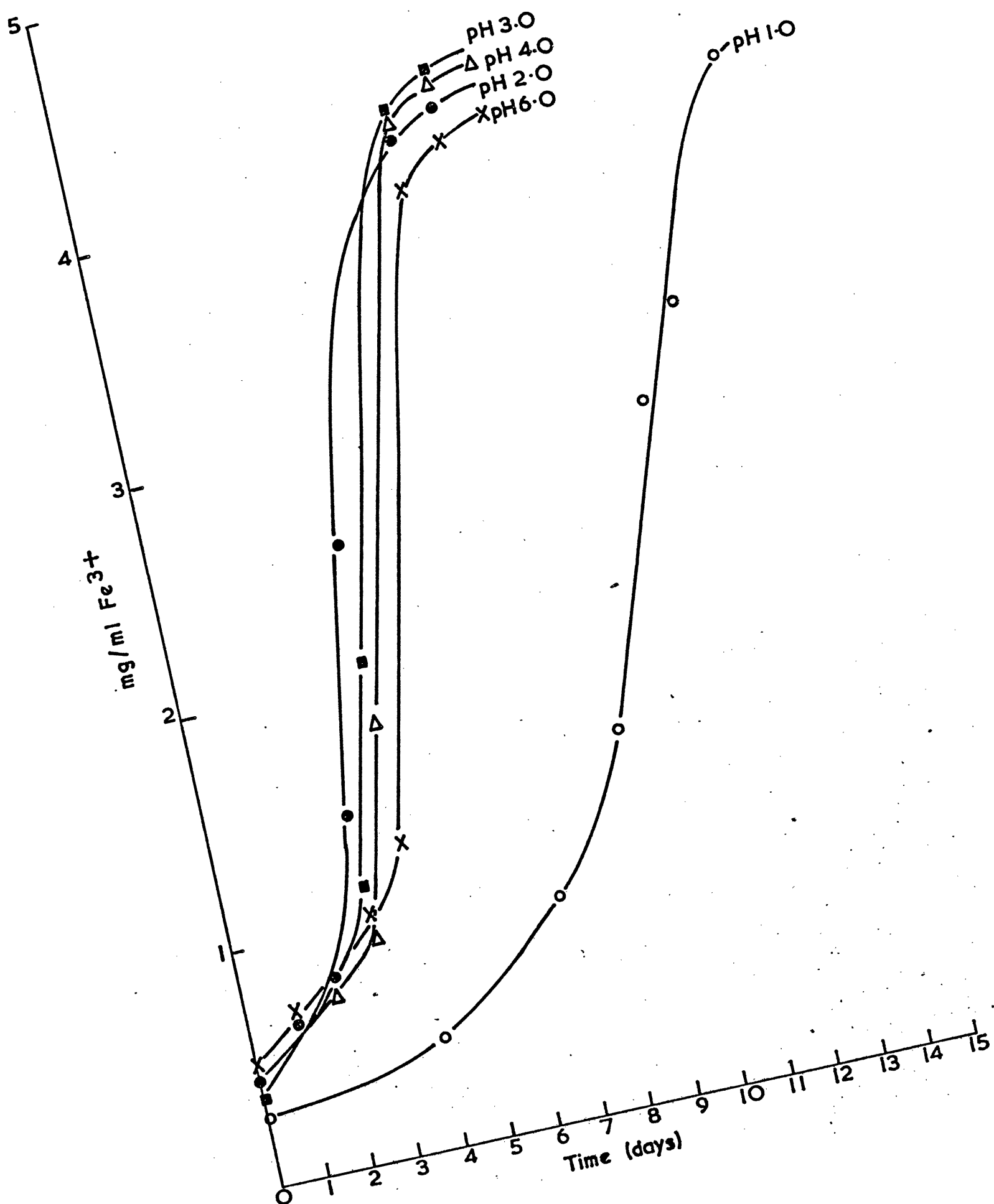
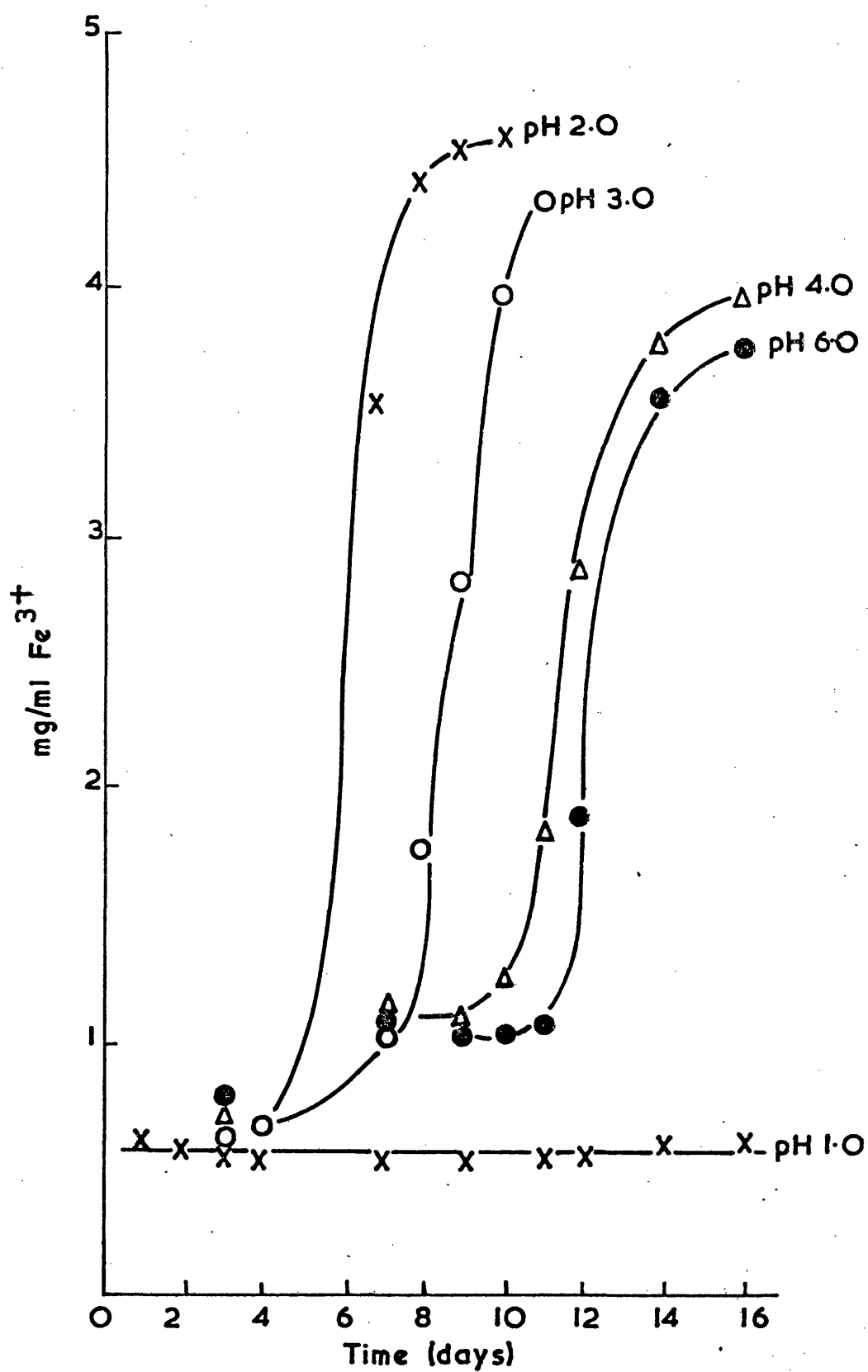


FIG.17

Effect of initial pH of medium on growth of T. ferro oxidans Tf 26

showed that at 30°C growth occurred at pH 1.8 and at the pH limit of normal growth, pH 1.5, after a short lag phase (Fig.18). When the temperature was 35°C (Fig. 19) growth occurred at pH 1.8 but very poor growth was achieved at pH 1.5. Brock and Dorland (1970) showed that in extreme ecological environments among thermophilic micro-organisms those capable of surviving in hot springs of 85°C - 90°C were in neutral or alkaline water, whereas in acidic condition of pH 2.0 - 3.0 the limits of microbial existence was 60°C - 65°C. The experiment with T.ferro oxidans showed that similar principles apply to mesophilic organisms in less extreme environments.

#### Summary of growth in a liquid ferrous-sulphate medium

Growth experiment with T.ferro oxidans in liquid ferrous-sulphate medium showed a remarkable lack of strain variation. Although results for just a few strains are given all strains were tested for growth rate in 'F' medium (25 mg/ml  $\text{FeSO}_4$ ) static at 30°C and 15°C and no significant variation could be found. The length of the lag phase depended on the age of the inoculum with the one exception of Tf26.

No evidence was found for the suggestion that strains of T.ferro oxidans become adapted to laboratory conditions. Three cultures of T.ferro oxidans which had been freshly isolated from ore samples from sites in Devon and had had only one growth cycle in 'F' medium were compared in growth rate to Tf3 at 30°C and 15°C. The results (Figs. 20 and 21) show no difference in growth rates between the 'native' and 'laboratory' strains.

FIG.18  
Effect of initial pH of medium at 30°C T.ferrooxidans Tf3

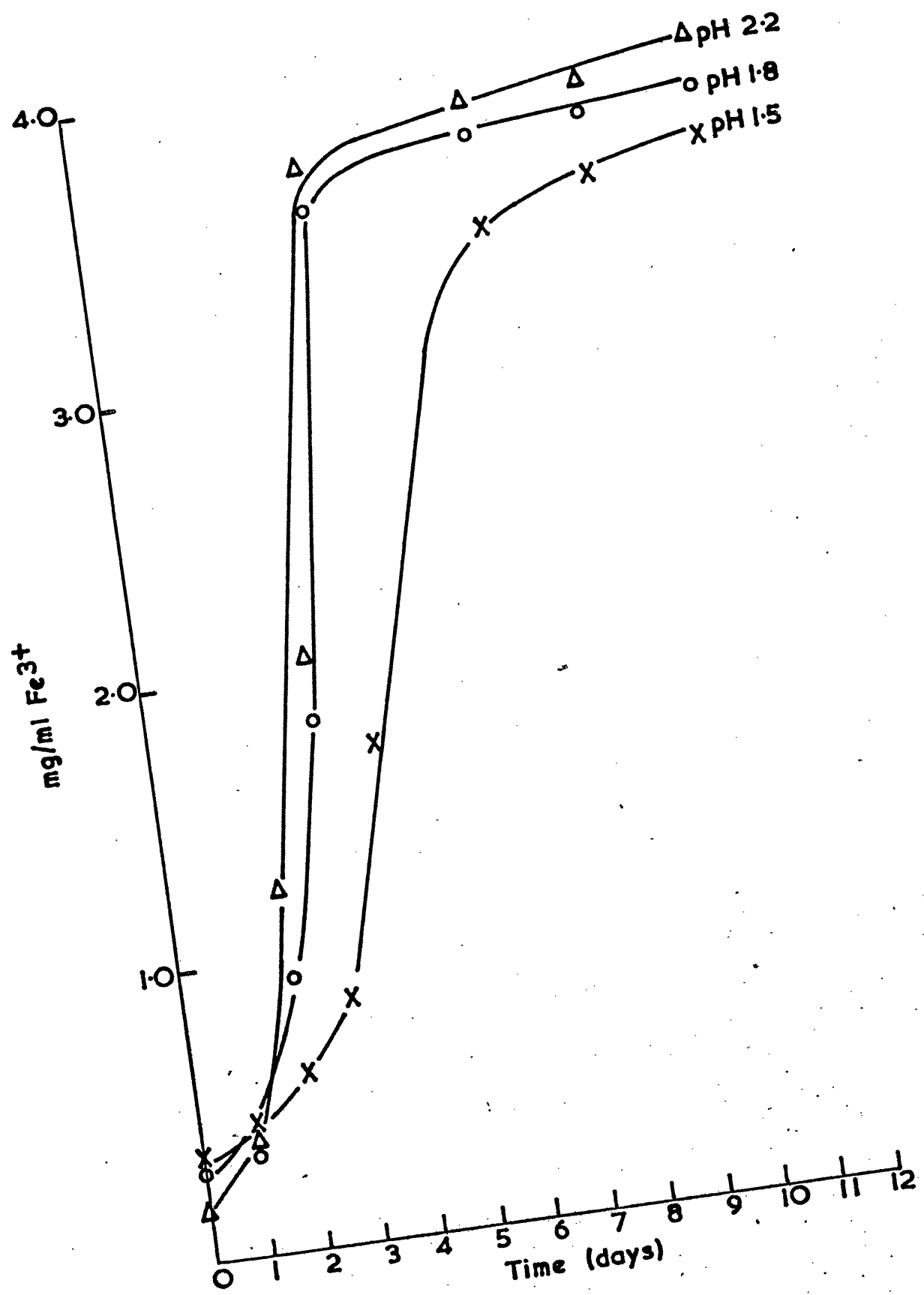


FIG. 19  
Effect of initial pH of medium at 35°C on *T. ferrooxidans* T13

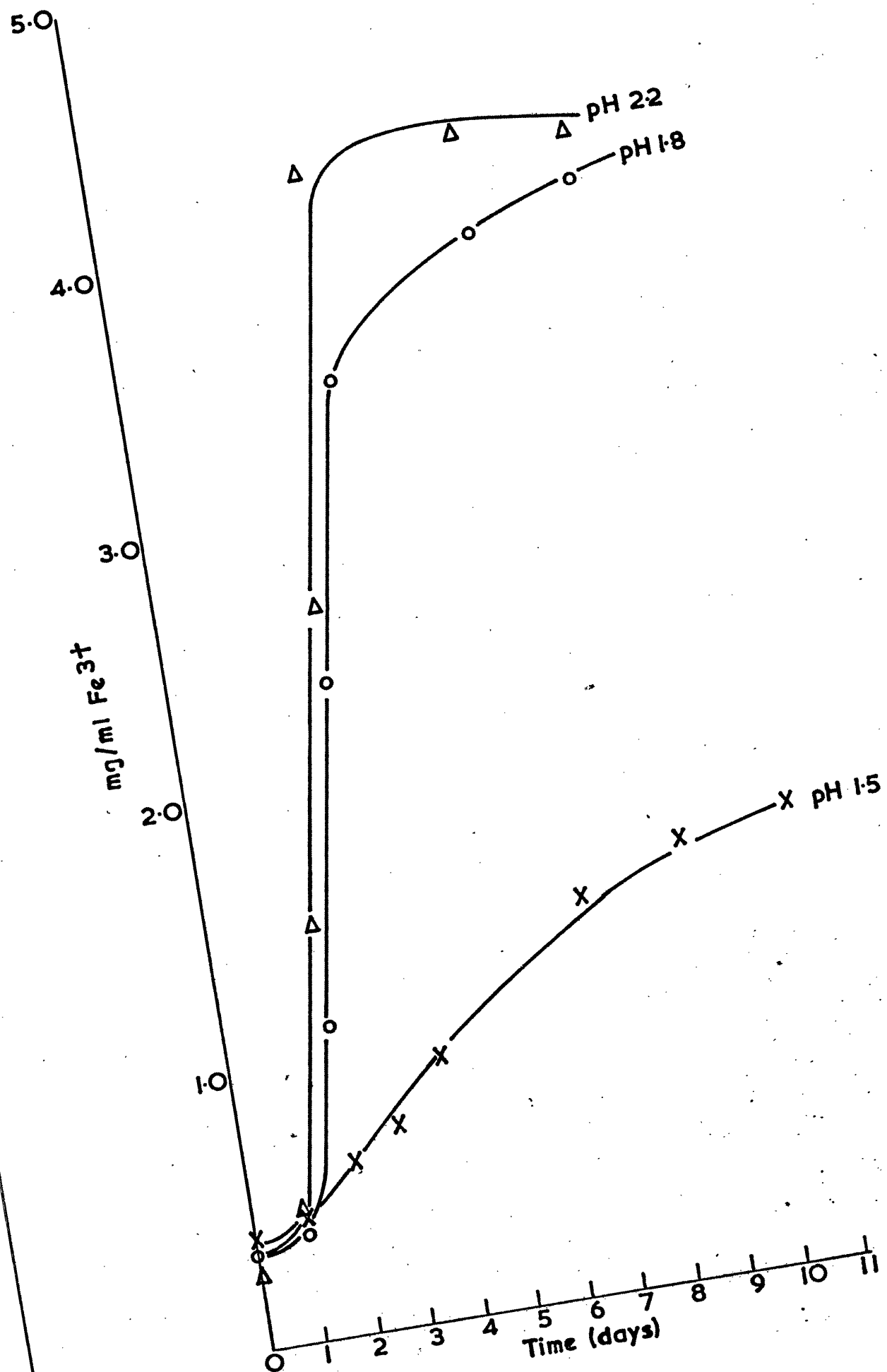


FIG. 20

Growth of newly isolated strains of T. ferro oxidans and Tf3 in ferrous sulphate at 30°

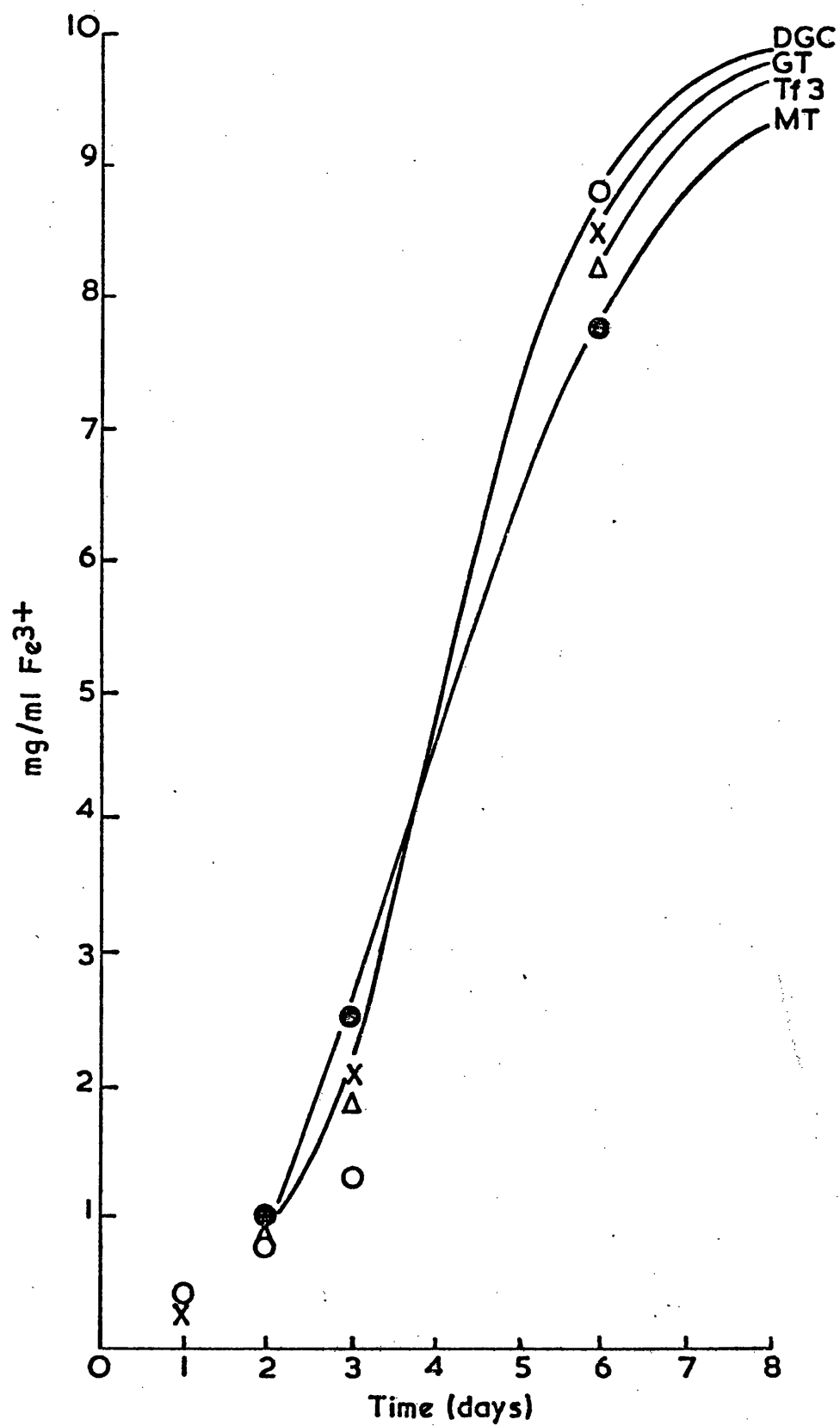
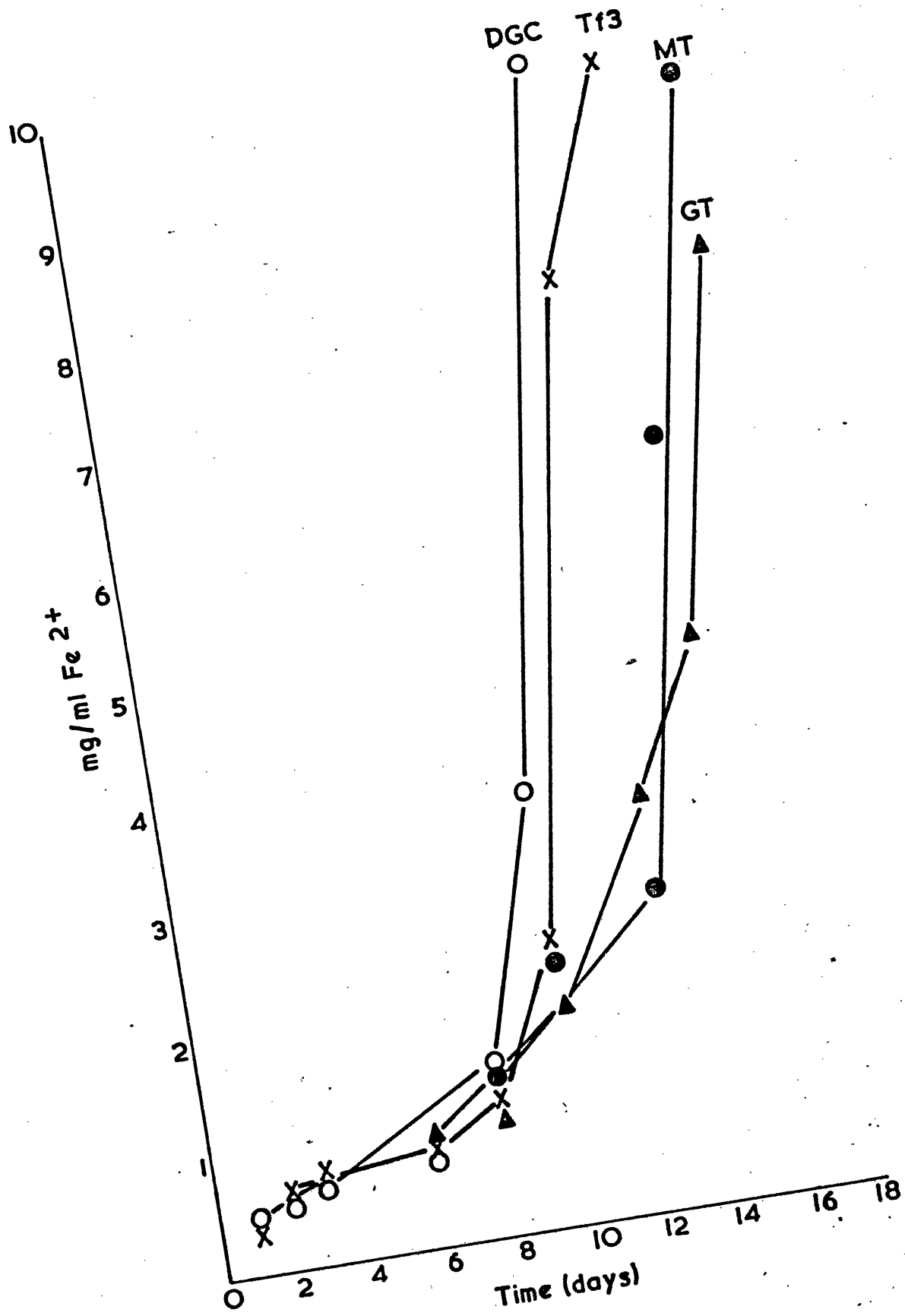




FIG.21  
Growth of newly isolated strains of T.ferro oxidans and Tf3 in  
ferrous sulphate medium at 15°C



Ferrous sulphate utilisation as estimated by the  $\text{FeCl}_3$ -HCl complex could be used as an estimate of bacterial growth in liquid cultures at  $30^\circ\text{C}$  or below in non-aerated cultures. Above  $30^\circ\text{C}$ , or with aeration, the appearance of ferric sulphate was not directly related to bacterial growth but was produced by a combination of bacterial growth and atmospheric oxidation of the ferrous sulphate. The degree of atmospheric oxidation of the ferrous sulphate in the experiments shown in Fig. 10 where Tf3 and Tf18 were growing in shaken cultures and in Fig. 13 (Tf3 growing at  $35^\circ\text{C}$ ) was not determined but great care must be taken in the interpretation of the appearance of ferric sulphate in a liquid culture in relation to the bacterial growth of that culture.

The peculiarities of *T.ferro oxidans* strain Tf26 both in liquid and on solid ferrous sulphate medium have been noted.

#### SECTION IV

##### Growth of *T.ferro oxidans* on pyrite and pyritic ores

Growth studies of *T.ferro oxidans* were initially restricted to growth on pyrite and pyritic ores. Two grades of pyrite were used; high purity (H.P.) pyrite was obtained from museum grade, hand picked, crystals and Tharsis pyrite (*T.pyrite*) was mined in Spain. A pyrite-containing ore mined at Elliot Lake in Canada (ELO) was also used. An analysis of the Tharsis pyrite and the ore from Elliot Lake was made by the Atomic Weapons Research Establishment at Aldermaston and is given in Table 10.

The ore samples received had been crushed to a convenient size (approx.  $\frac{1}{2}$  inch lumps). These were treated in an agate ball mill and sieved. The fraction passing a 200 mesh but retained by a 300 mesh sieve was used. This fraction contained particles of 45  $\mu\text{m}$  - 70  $\mu\text{m}$  diameter. The dry milled ore was sterilised by autoclaving at 10 lb for 15 minutes.

There are two basic problems in the estimation of bacterial growth in liquid culture when pyrite or pyritic ore is the energy substrate. The first is that a viable count is meaningless because *T.ferro oxidans* adsorbs to pyrite particles from which they cannot be removed by vigorous shaking so that the number of free bacteria in the solution are not representative of the total number of bacteria present (see Section II).

The second problem of estimating bacterial growth lies in the complex set of reactions which occur when bacteria grow on pyrite. These are described fully in the introduction. Once

TABLE 10Analysis of Tharsis Pyrite

	%
Fe	42.4
Sulphide S	44.1
Sulphate S	0.9
Zn	5.0
Pb	0.5
Cu	0.75
Ca	0.1
As	0.3
SiO <sub>2</sub>	3.75

Analysis of Elliot Lake Ore

	%	ppm
Fe	2.75	
Zn		100
Na		1000
Cu		200
Pb		1000
AL	5.0	
Mg		2000
Ma		100
Ba		500
Ca		200
K	2.5	
Total S	2.62	

The analyses were carried out by the Atomic Weapons  
Research Establishment at Aldermaston.

growth is initiated the ferric sulphate produced will react with the pyrite to give ferrous sulphate. Estimations of soluble iron as ferric, or total, iron reflect both the chemical and bacterial reactions which are taking place. However, bacterial growth was estimated by the production of ferric sulphate as this is more indicative of bacterial activity than viable counts from pyrite suspensions.

#### Comparison of T.pyrite, H.P.pyrite and ELO as substrates for growth

For growth experiments the pyrites and pyritic ores were used at a 1% (weight for volume) concentration. The milled ore (300 mesh) was weighed and 1.0g placed in a 500ml flask. The flask and its contents were sterilised by autoclaving. When cool, 100ml of sterile medium was added and the flask inoculated and weighed. Each 2ml sample taken during the experiment was centrifuged at 5000 rpm for 5 minutes to remove pyrite and the supernatant assayed. Full details are given in 'Methods and Materials'.

A comparison of the growth of I.ferro oxidans strains Tf3 and Tf26 on 1%(w/v) suspensions of T.pyrite, H.P.pyrite and ELO in either 'F' basal salts or glass distilled water pH2.2 (0.005M H<sub>2</sub>SO<sub>4</sub>) is made in Figs. 22, 23 and 24. Pyrite is the natural source of energy for these organisms but as may be seen in Fig.22 even in the presence of 'F' salts growth is limited by the availability of energy from the substrate. The fastest 'growth rates' were achieved with T.pyrite where 3.2 mgs/ml ferric sulphate were solubilised in 10 days and over 4.0 mgs/ml in 100 days at 30°C in 'F' salts. The solubilisation of iron from H.P.pyrite was slower and in 'F' salts only 2.8 mgs/ml ferric iron were found in

FIG.22

Comparison of the growth of T.ferro oxidans strains Tf3 & Tf26  
on 1% Tharsis pyrite

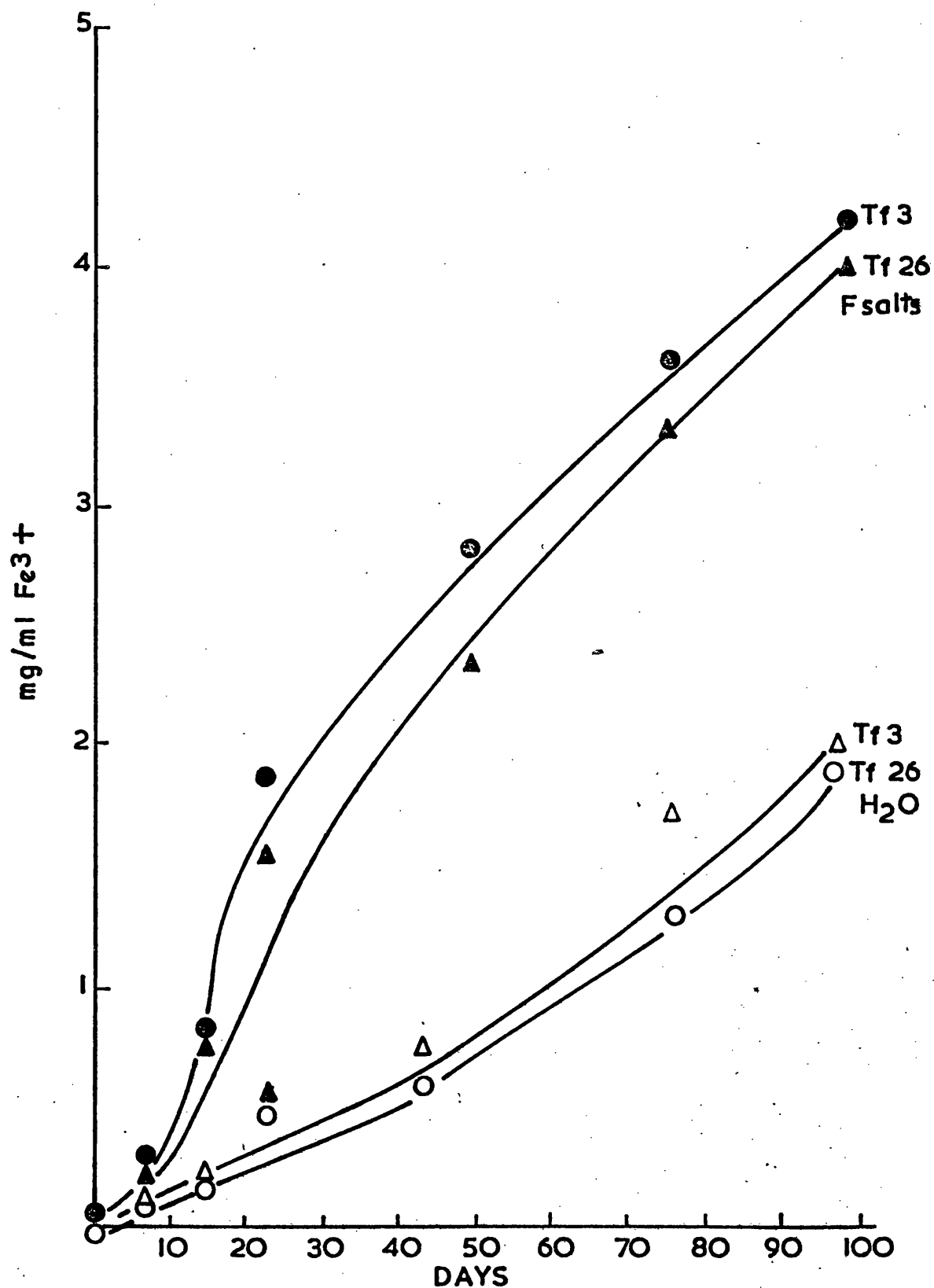


FIG. 23

Comparison of the growth of T. ferro oxidans strains T1 3 & T126  
on 1% H.P. pyrite

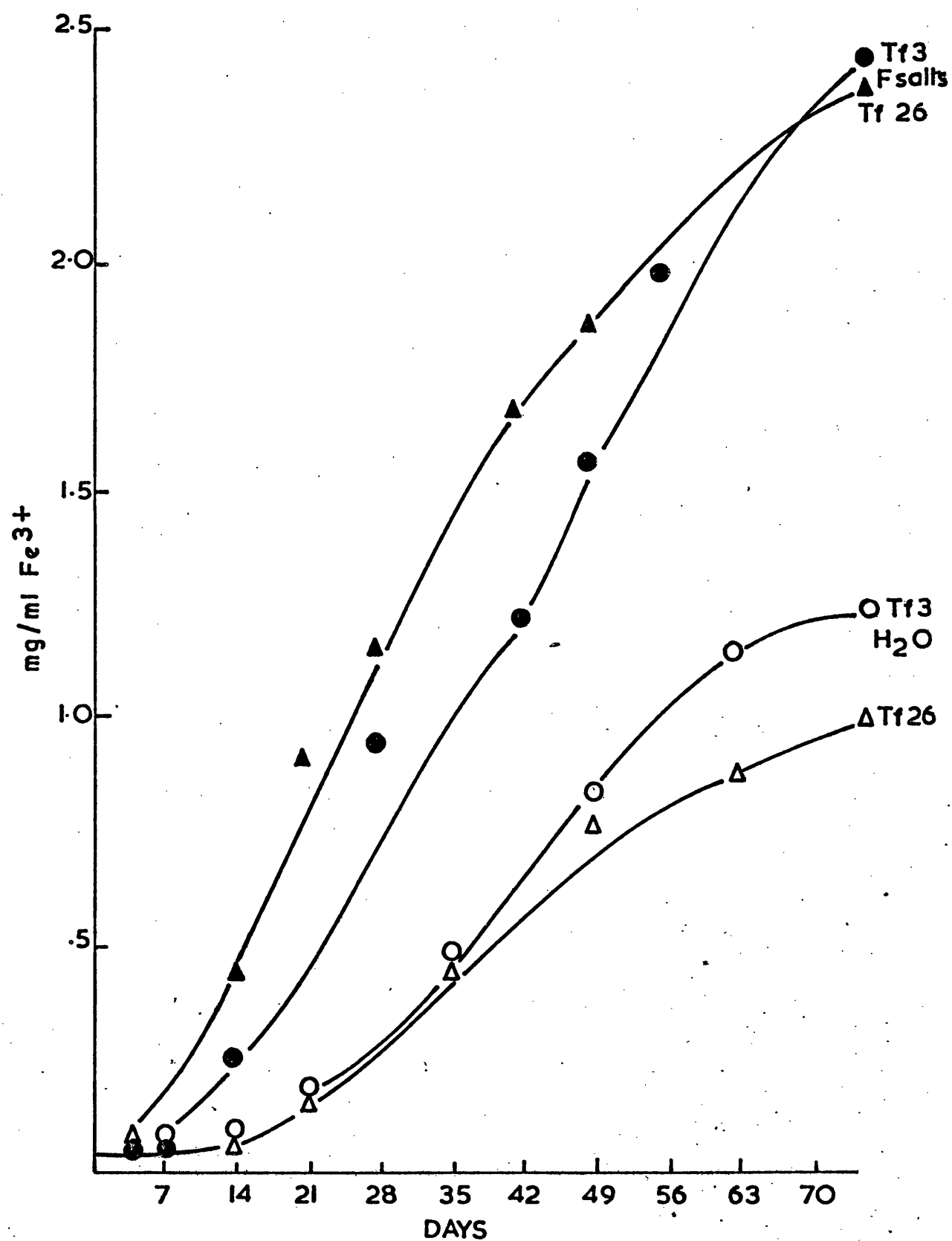
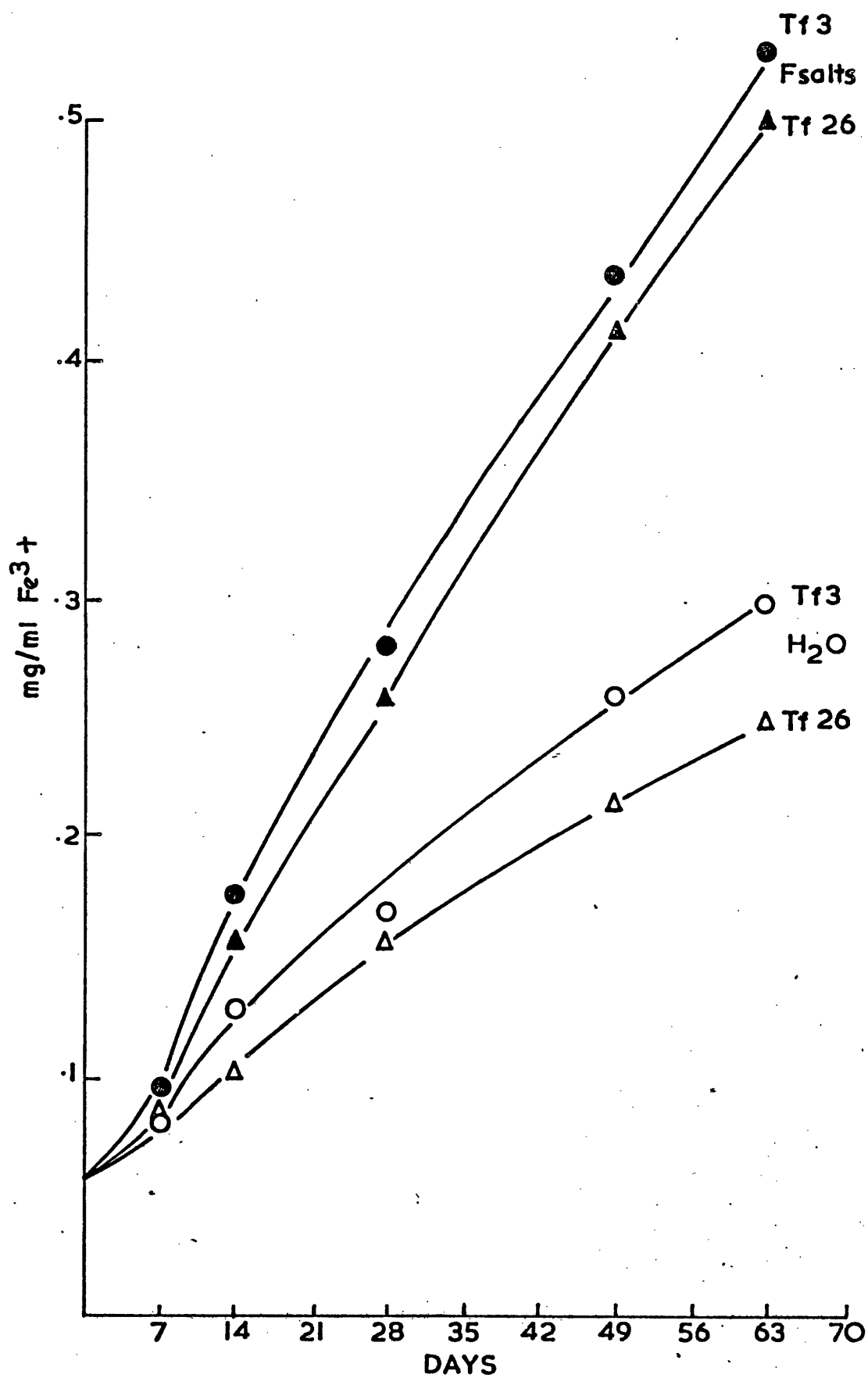


FIG. 24.

Comparison of the growth of T. ferro oxidans strains Tf3 & Tf26  
on 1% Elliot Lake ore





70 days (Fig. 23). The poorest growth was recorded with ELO where in 70 days with 'F' salts only 0.53 mg/ml ferric iron was found. Growth of Tf26 on pyrite was very similar to that of Tf3.

The comparative growth rates in 'F' salts and 'water' ( $0.005\text{MH}_2\text{SO}_4$ ) show that other mineral deficiencies are limiting growth. When T.pyrite was the energy substrate 4.2mg/ml ferric iron was produced in 'F' salts in 100 days whereas in 'water' less than half this amount was produced (1.9mg/ml). If the components of the 'F' salts (Table 10) are added to the 'water' individually or in groups it was shown (Fig. 25) that the addition to the growth medium of Mg and  $\text{PO}_4$ , as  $\text{MgSO}_4$  and  $\text{K}_2\text{HPO}_4$ , at the concentration used in F, stimulated iron solubilisation but the addition of KCl to the Mg and  $\text{PO}_4$  had little further effect. Growth was still limited and the deficient nutrient was nitrogen. Although the liquid medium was acidic and ammonia was being absorbed from the atmosphere, its rate of absorption was not sufficient for maximal growth on the energy available as shown by comparative growth in 'F' salts. Similar results were found with H.P. pyrite and ELO.

#### Acid production in pyrite media and its effect upon growth

As the growth of T.ferro oxidans proceeds on T.pyrite sulphuric acid is produced and the pH drops. Fig. 26 shows the solubilisation of ferric sulphate with the associated drop in pH during growth of T.ferro oxidans strains Tf3, Tf18 and Tf26 in 'F' T.pyrite medium at  $30^\circ\text{C}$ . All cultures were initially at pH 2.2. Growth proceeds for 56 days by which time 4.8mg/ml - 5.3mg/ml ferric iron had been produced and the pH of the medium was pH 1.4 - 1.5.

Growth of T.ferro oxidans strain Tf3 on 1% Tharsis pyrite in  
Various liquid media

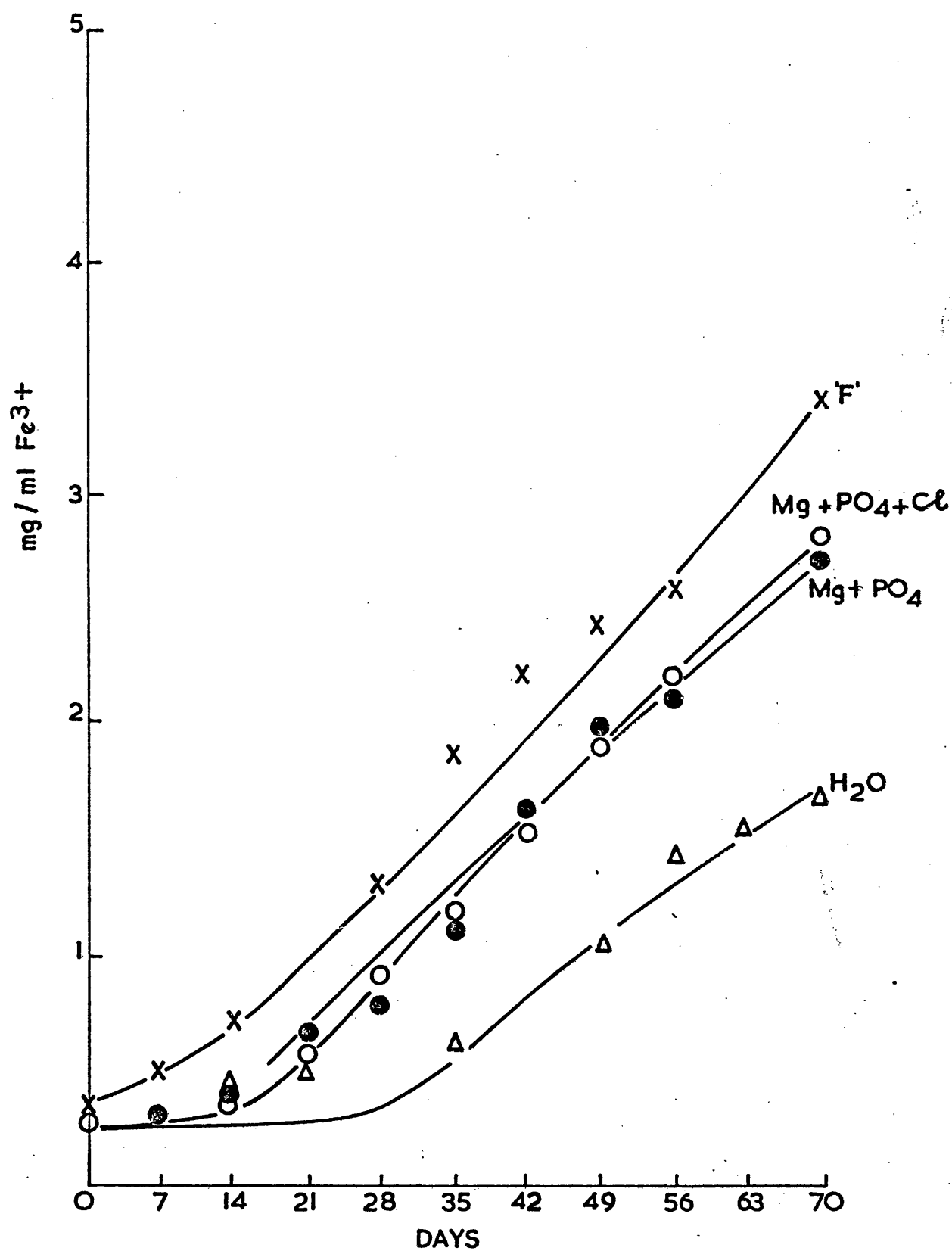
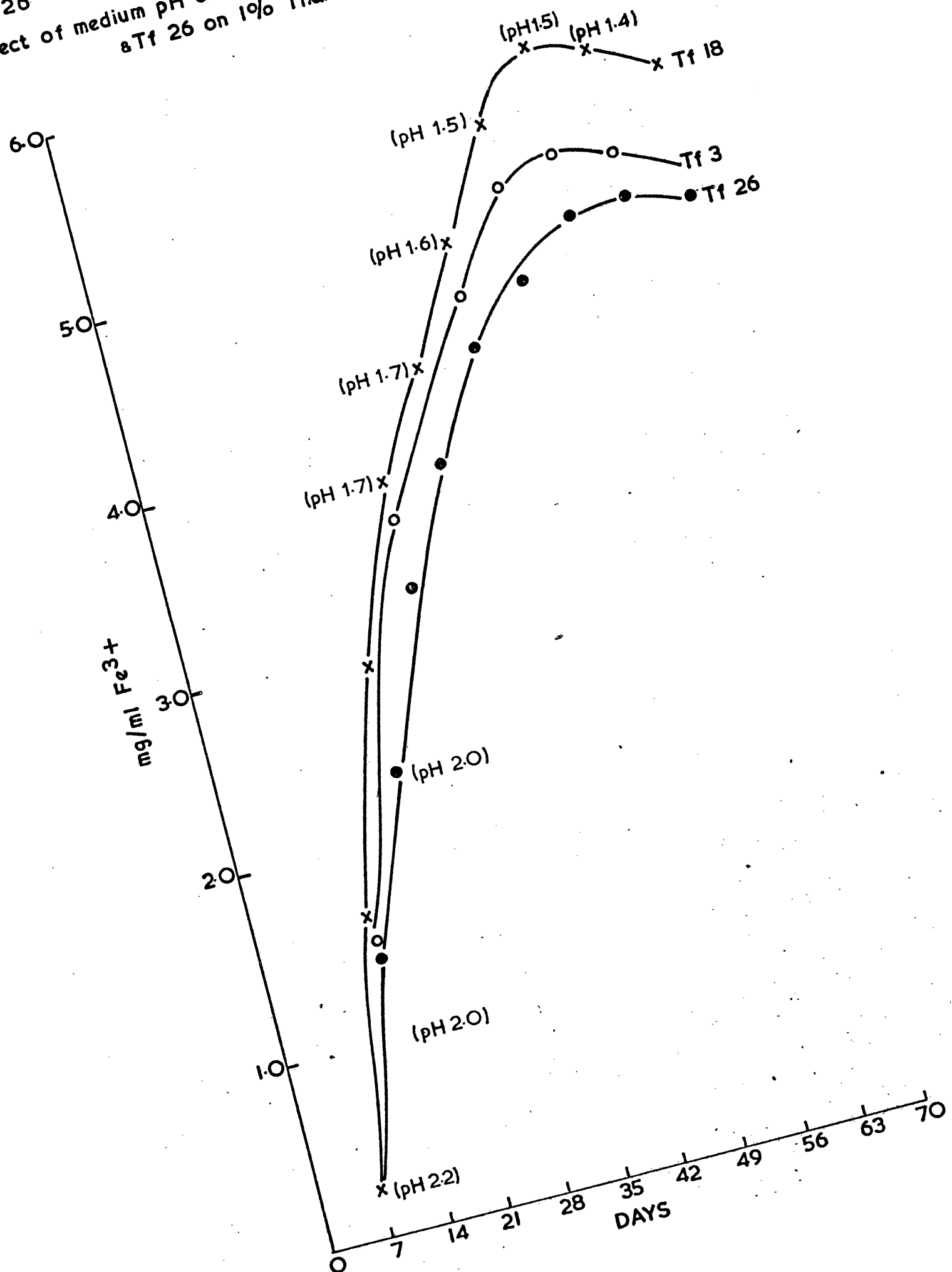


FIG. 26  
Effect of medium pH on growth of *T. ferro oxidans* Tf 3, Tf 18,  
Tf 26 on 1% Tharsis pyrite



At this time growth appeared to stop and the medium was depleted of ferric iron presumably due to its chemical reaction with pyrite. The pyrite substrate had not been exhausted and results from experiments on ferrous sulphate medium would suggest that the acidity of the medium had limited bacterial growth.

When other newly isolated cultures of T.ferro oxidans were tested for growth on T.pyrite in 'F' salts certain cultures were found which did not stop growing at pH1.5 but continued to grow as shown in Fig. 27. The pH drop was the same in all cultures but whereas Tf3 reached a maximum growth at pH1.5, when production of ferric iron ceased and the medium became depleted, the other cultures continued to produce ferric iron although the rate decreased. This effect was not confined to growth on T.pyrite but was also observed with growth on ELO (Fig. 28). Tf3 and Tf18 showed a similar growth pattern with a restriction on ferric sulphate production initiated at pH1.5 whereas Tf52 and Tf74 continued to grow.

#### Study of fast pyrite utilising strains

The fast pyrite utilising strains T.ferro oxidans Tf49, Tf52, Tf54 and Tf74 were tested for growth rates on ferrous sulphate medium, (Fig. 29), which were found to be similar to that of Tf3. It would seem unlikely therefore, that the increased rate of ferric sulphate production from pyrite was simply related to a shorter bacterial generation time. Closer examination of these newly isolated cultures showed that they were all mixed autotrophic culture of at least T.ferro oxidans and T.thio oxidans. No heterotrophic bacteria could be isolated from these cultures. The cultures Tf49, Tf52 and Tf54

Comparison of the growth of newly isolated cultures T149 & T152, T174 with strain T13 on 1% Tharsis pyrite

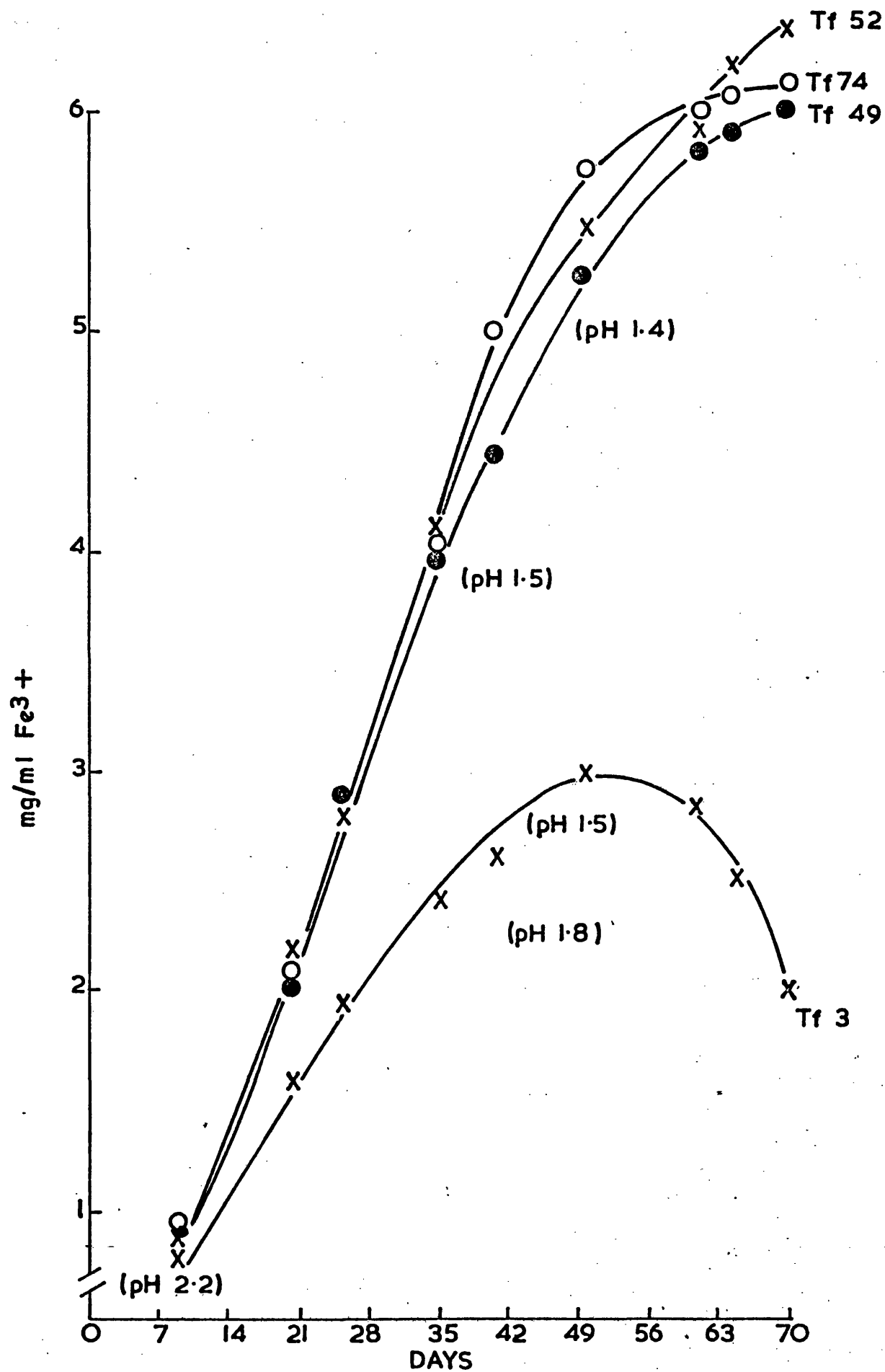


FIG.28

Growth of T ferro oxidans, Tf 52, Tf 74, Tf 18, & Tf 3 in 5%  
Elliot Lake ore

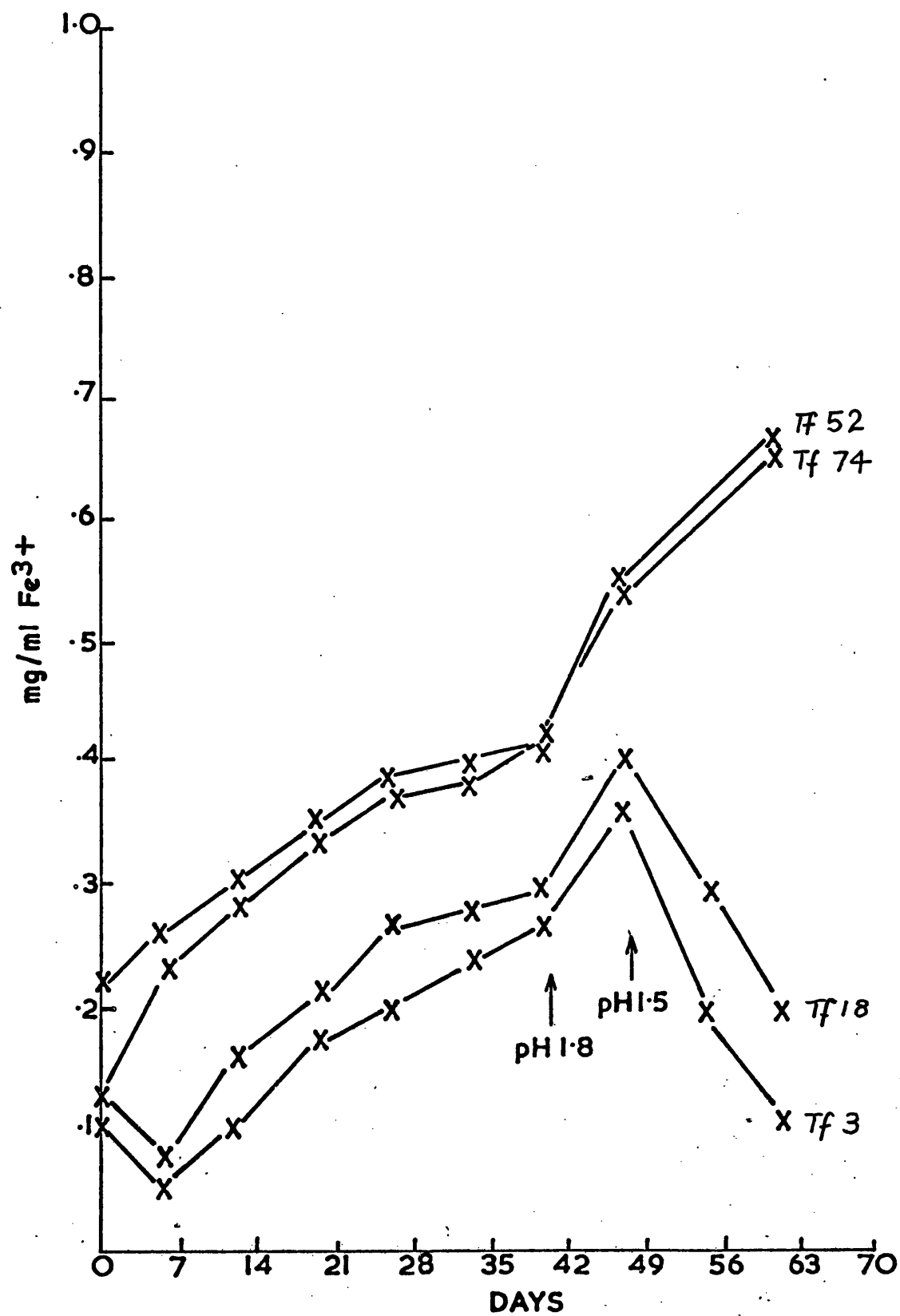
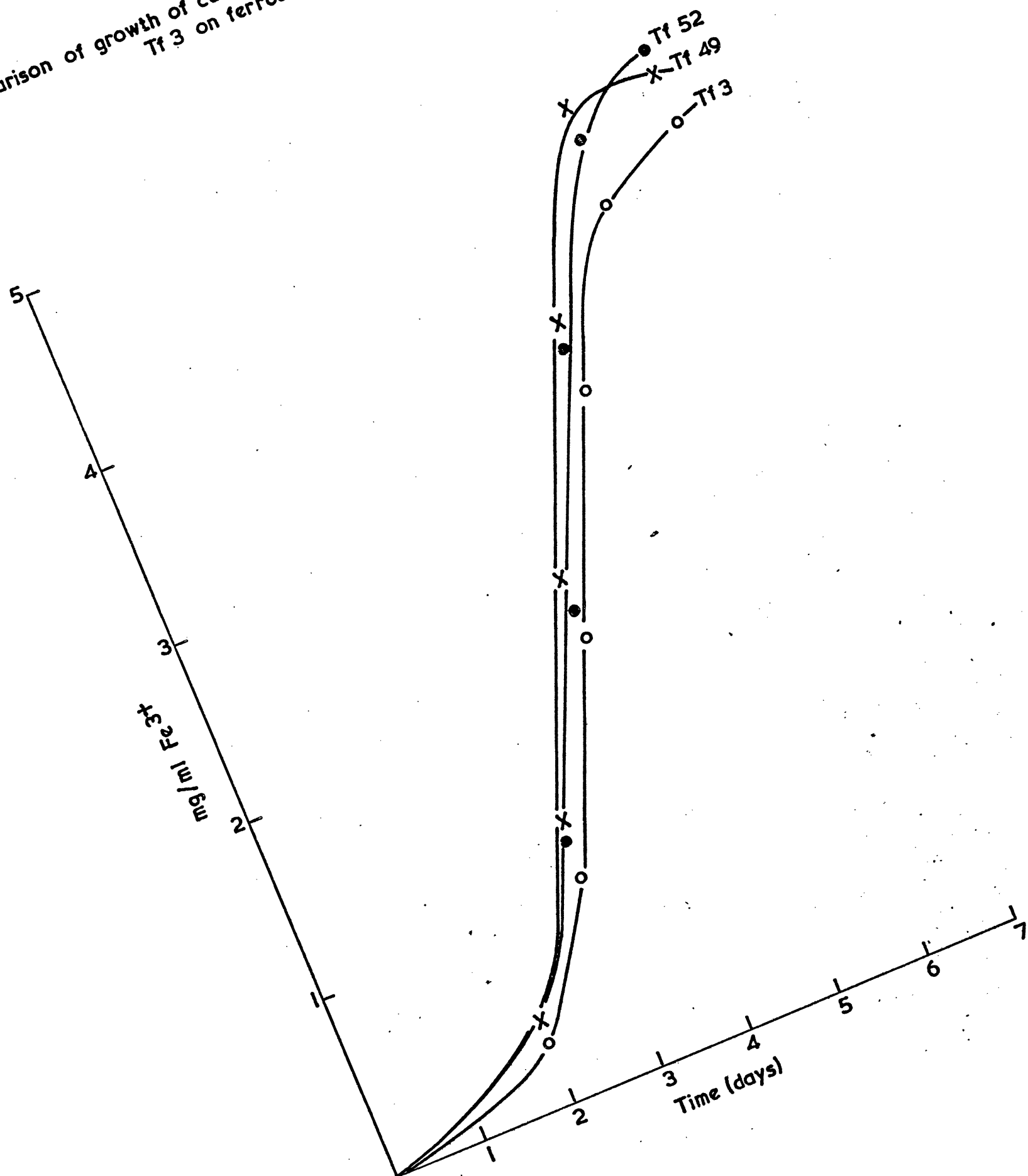


FIG.29  
Comparison of growth of culture Tf 52 & Tf 49 with T.ferrooxidans  
Tf 3 on ferrous sulphate medium

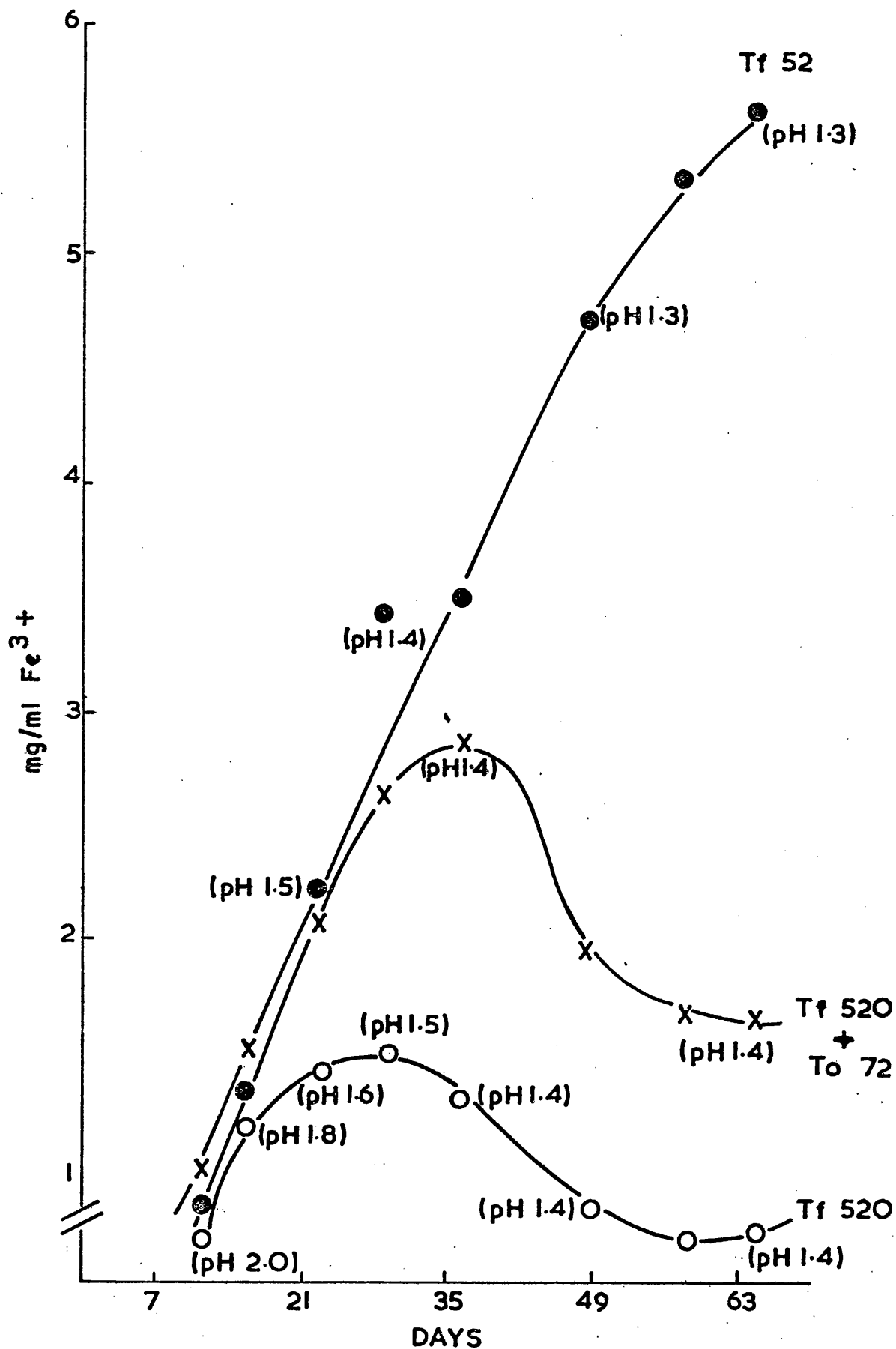


were originally isolated, in ferrous sulphate medium, from acid mine-water samples from a coal mine in Scotland. Tf74 was isolated, also in ferrous sulphate medium from a sample of Tharsis pyrite which had been crushed, milled and sieved but not sterilised.

Attempts were made to separate the bacteria in these mixed cultures and the Thiobacilli component bacterial strain purified from single colonies by repeated isolation on 'F' agarose medium for T.ferro oxidans and on thiosulphate agarose medium for T.thio oxidans. T.ferro oxidans gave a brown precipitate of ferric sulphate within and around the colonies, only one colony type was found and the bacterial cells were non-motile. T.thio oxidans gave one colony type on thiosulphate agarose medium, no growth, as indicated by a ferric sulphate precipitate, was found in ferrous sulphate medium and the bacteria were freely motile in sulphur and thiosulphate medium. Culture Tf52 was isolated into T.ferro oxidans Tf520 and T.thio oxidans To 72. The results of growth of these cultures on T.pyrite both separately and mixed together were most interesting (Fig. 30). T.ferro oxidans Tf520 had grown quite poorly and by day 31 when only 1.5mg/ml ferric iron had been solubilised the pH was 1.5 and ferric iron production declined. The mixture Tf520 and To72 grew better and in 31 days had solubilised 2.6mg/ml ferric iron however the pH began to drop to pH1.4 and after day 40 ferric iron was being taken from the medium. The original mixed culture Tf52 grew well and by day 40 at pH1.4 had solubilised 3.5mg/ml ferric iron. Growth was not inhibited and increased. A slight decline in the rate of ferric production was seen from day 50 when at pH1.3 4.3mg/ml ferric iron was present. The experiment was terminated on day 66 when 5.6mg/ml ferric iron was



Growth of culture Tf 52, T ferro oxidans Tf 52O and T. thio oxidans To 72 on 1% Tharsis pyrite



present and the pH unchanged at pH1.3. These results suggest that the original culture may have contained other, as yet unidentified bacteria, which play an important role in pyrite utilisation.

Alternatively T.ferro oxidans or T.thio oxidans, or both, lost some critical ability during purification or possibly the ratio of one to the other is critical and was not achieved in this experiment.

Although the reason could not be identified the results showed that the mixed culture was able to utilise T.pyrite at a much faster rate, for a longer time and with less sensitivity to pH than either the pure T.ferro oxidans Tf520 or the mixture with T.thio oxidans To72.

#### Summary of growth on pyrite and pyritic ores

Growth studies of T.ferro oxidans on pyrite and pyritic ores showed that growth did occur but the rate was limited by the available energy substrate even in the presence of 'F' salts. If water replaced the salts, as would be more natural in an ecological situation, the growth rate was even more limited due to the absence of additional growth requiring substances especially nitrogen. Growth was best on a pyrite rich ore like Tharsis pyrite and poorest on a low pyritic ore like Elliot Lake. The production of sulphuric acid during growth on pyrite was found to restrict the growth of many strains and in particular pure strains of T.ferro oxidans. Mixed autotrophic cultures were shown to utilise the pyritic substrate with greater efficiency and tolerate more acid conditions. Although T.ferro oxidans were isolated from the mixed culture we do not know if other organisms, especially the acidophylic thiobacilli like T.acidophilus and T.intermedius were also present.

The close association of T.ferro oxidans and T.thio oxidans has long been noted (Colmer and Hinkle, 1947) and the reason for the presence of T.thio oxidans was thought to be due to its ability to use the free sulphur but the results from growth experiments of mixed cultures on pyrite suggest that T.thio oxidans may be playing a far more important role in pyrite utilisation. The survival of T.thio oxidans through many subcultures in ferrous sulphate medium is known to occur but has not been accounted for.

A greater understanding of these mixed cultures is required before the bacteriological process of pyrite utilisation can be established.

## SECTION V

### Nitrogen fixation by I.ferro oxidans

The ability of I.ferro oxidans to fix atmospheric nitrogen has never been considered seriously because claims that growth was possible in the absence of a soluble nitrogen source were disregarded and explained as due to absorption of ammonium ions from the atmosphere by the acid media.

If 75ml ferrous sulphate medium pH2.2 was incubated in a 250ml flask sealed with a cotton wool plug for 14 days at 30°C and the ammonia absorbed after this time estimated by titration with H<sub>2</sub>SO<sub>4</sub> it was found that 0.364 µgm/ml ammonia had been absorbed. If the flask was sealed with a rubber 'Suba-seal' (W. Freeman & Co.Ltd.) 0.163 µgm/ml ammonia was absorbed. This experiment, with flasks inoculated with I.ferro oxidans, showed that ammonia present in the cotton wool plugged flask after 14 days was 0.154 µgm/ml and in the sealed flask 0.064 µgm/ml but all the ferrous iron had been utilised in both flasks. In the plugged flask as ammonia was taken out of solution by the bacteria more would be absorbed from the atmosphere; in the sealed flask there was not sufficient ammonia to provide all the nitrogen required to support the bacterial growth and iron utilisation that had occurred.

### Biological nitrogen fixation

The biological fixation of atmospheric nitrogen was first recognised in the late 1890's but only recently has the economic importance to agriculture been recognised. The one time accepted

idea that growth of an organism on medium to which combined nitrogen had not been definitely added provided reportable evidence of fixation was soon discarded as the difficulty, if not impossibility of preparing truly N-free medium was appreciated. Claims, however, of organisms capable of fixing nitrogen have been unlimited from bacteria, yeasts and fungi to man but with improved techniques many such claims have been disproved and many yeasts and filamentous fungi shown to be very efficient nitrogen scavengers and able to grow on minimal amounts of ammonia and other forms of combined nitrogen present in the air which can provide a continuous supply at a very low level. The ability to fix atmospheric nitrogen is at present restricted to certain bacteria.

The isotopic tracer method for estimating fixed nitrogen using a mass spectrometer was introduced by Burns and Miller (1941) and was a more accurate estimation than total nitrogen by the Kjeldahl method. It is the definitive test for nitrogen fixation.

The enzyme associated with fixation, the nitrogenase was isolated (Carnahan, Mortenson, Mower and Castle 1960) and studied in cell-free extracts. It was demonstrated to be a most versatile reducing catalyst and the ability to reduce acetylene to ethylene (Schollhorn and Burris 1966; Dillworth, 1966) among other things, was applied to a very simple but sensitive assay system as will be described.

In a comparative biochemistry of nitrogen fixation P.W. Wilson (1952) arranged the organisms capable of this reaction according to certain physiological characteristic, heterotrophic,

autotrophic or photosynthetic; free-living or symbiotic; aerobic or anaerobic. There was a prominent gap in the table for an aerobic autotroph. A gap that can now be filled by T. ferro oxidans.

#### Acetylene reduction as presumptive evidence of nitrogen fixation

The acetylene reduction test is widely used to demonstrate the presence of nitrogen-fixing enzymes in micro-organisms (Hardy, Holsten, Jackson and Burns, 1968; Postgate, 1970a). It is based on the observation of Dilworth (1966) and Schollhorn and Burris (1967) that preparations of nitrogenase enzyme reduce acetylene specifically to ethylene which can be detected with extreme sensitivity by gas-liquid chromatography (GLC).

The biological nitrogen-fixing systems which have been studied show certain similarities (Postgate, 1970b). The nitrogenase is irreversibly damaged by oxygen (Drozd and Postgate, 1970) and repressed by growth in media containing ammonium ions (Strandberg and Wilson, 1968). Molybdenum was found to be associated with the enzyme fraction (Mortenson, 1966). These facts were taken into consideration in the design of acetylene reduction tests for T. ferro oxidans. The medium used was 'F' medium from which all soluble nitrogen had been omitted (N-free F). Molybdenum was added as  $\text{Na Mo O}_4 \cdot 2\text{H}_2\text{O}$  0.002%(w/v). To prevent ammonia from being absorbed from the atmosphere the experiments were carried out in flasks which were sealed with 'Suba-seals'. The seals have the great advantage that they can be punctured by a hypodermic needle and therefore additions or extractions of samples of gas or culture fluid could be easily made. Any air that was added to the experimental flasks was first scrubbed of ammonia through concentrated  $\text{H}_2\text{SO}_4$ .

Experiments were carried out in 50ml flasks the actual volume of which was between 62.7ml and 67.3ml. Each flask contained 15ml of N free F medium giving a gas volume of approx. 50ml. The flasks were inoculated and gassed with  $N_2 + 5\% CO_2$  allowing sufficient to flow through the flask to displace the air. The flasks were sealed with 'Suba-seals' and 7ml of the atmosphere in the flask replaced by 7ml of ammonia-free air. The flasks were incubated static overnight in a  $30^\circ$  waterbath. The details of the acetylene, ethylene assay are those given by Postgate (1972) as is the production of acetylene from calcium carbide and water. After overnight incubation one ml of acetylene was added to each flask and incubation, with gentle shaking, was continued for 4-7 days. Gas samples of 1ml were removed for analysis by GLC. Details of the GLC method are given in 'Methods and Materials.' In the initial studies the quantities of ethylene produced were measured by peak heights which were multiplied by the degree of attenuation required to register that peak on the chart. A more accurate estimate of ethylene production was made with a Kent chromatogogue integrator in conjunction with the GLC which gave peak areas. These were calibrated using known dilutions of 99% pure ethylene (Postgate, 1972) and the quantities of ethylene produced were recorded as nanomoles/ml.

A small amount of ethylene was found as an impurity in the acetylene produced from calcium carbide and water. One ml of acetylene was added to each test flask, approx. 50ml, and a 1ml aliquot of this dilution gave an ethylene peak height on the GLC of 5 - 15mm at an attenuation of 10. For a result to be considered indicative of acetylene reduction on ethylene peak requiring an attenuation of at least x 50 must be obtained.

Table 11 shows the quantities of ethylene produced by five strains of I.ferro oxidans over a period of 4 days. The acetylene was added at 10.00 on day 1; at 16.00 strains Tf8 and Tf9 were already showing increases in the ethylene peaks. By day 2 all strains showed significant increases in the amounts of ethylene present in each flask. This continued to increase during the next 24 hours. Incubation after day 3 showed no further increase in ethylene production which was possibly due to all the iron being utilised.

To be certain that the acetylene reduction was caused by the viable bacteria and was not the result of chemical reactions caused by the acidity of the medium a series of controls was set up. These consisted of uninoculated N-free F medium at pH1.0 and pH2.2; controls to test the effect of bacterial growth in the medium were set up from a 3-day culture of Tf3 from which the bacteria were either removed by Millipore filtration or killed by boiling the culture for 15 minutes. The controls were gassed and treated exactly as test cultures. Acetylene was added and samples taken daily for 7 days and run on the GLC. No evidence of acetylene reduction was found in any control.

#### Effect of a soluble nitrogen source on acetylene reduction

Repression of a presumed nitrogenase by a soluble nitrogen source is considered good evidence of true nitrogen fixing.

I.ferro oxidans Tf3 was inoculated into N-free F, N-free F + 3.4mM  $(\text{NH}_4)_2\text{SO}_4$  and N-free F + 1.5mM  $\text{KNO}_3$  and the cultures tested for acetylene reduction over 6 days incubation at 30°C. The integrator was used and the results (Table 12) expressed in nanomoles/ml show that both ammonium and nitrate ions repress the enzyme responsible for the reduction of acetylene to ethylene in I.ferro oxidans.



TABLE 11

Ethylene production from acetylene by strains of I.ferro oxidans measured as peak heights (ph) in millimetres x attenuation (att)

Day	Tf3 ph x att		Tf1 ph x att		Tf8 ph x att		Tf9 ph x att		Tf21 ph x att	
0	7	10	6	10	9	10	6	10	6	10
0.25	12	10	6	10	22	10	21	10	12	10
1	28	200	31	100	110	100	88	100	40	100
2	88	200	30	200	70	200	68	200	32	200
3	100	200	21	200	62	200	70	200	35	200

TABLE 12

Ethylene produced from acetylene by I.ferro oxidans strain Tf3 in various media.

Day	N-free F n.moles/ml	N-free F + (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> n.moles/ml	N-free F + KNO <sub>3</sub> n.moles/ml
0	0.020	0.018	0.019
1	1.246	0.029	0.016
2	3.851	0.031	0.013
3	6.283	0.028	0.015
4	6.501	0.022	0.035
6	6.824	0.027	0.031

The amount of ammonium ions in the atmosphere available for absorption by an acid medium can be very critical for the repression of the nitrogenase. The laboratory at MRE can be considered 'normal'. Ammonia and ammonium reagents are used in the laboratories. At the ARC unit of Nitrogen fixation at the University of Sussex every care is taken to minimise the ammonium ion concentration in the laboratory atmosphere. If the first acetylene reduction experiment on T.ferro oxidans had not been done at this Unit it is quite possible that the nitrogen-fixing ability of T.ferro oxidans would have been overlooked because if a culture of T.ferro oxidans is set up in N-free F medium and incubated overnight with a cotton wool plug before being gassed, sealed and tested for acetylene reduction enough ammonia was absorbed at MRE to repress the enzyme. However, incubation at the ARC Unit did not provide sufficient ammonium ions to the medium and subsequent acetylene reduction could be shown. Initially acetylene reduction had been shown to occur at the ARC Unit but could not be repeated at MRE until it was found that the culture had to be isolated from the ammonia-rich atmosphere from the time of inoculation.

#### Acetylene reduction by presumed mixed cultures

Tsuchiya, Trivedi and Schuler (1974) claimed that acetylene reduction by culture of T.ferro oxidans was due to a 'microbial mutualism' in that the culture contained T.ferro oxidans and other bacteria such as Beijerinckia or Azotobacter which are known nitrogen fixers. If this were the case it should be possible to isolate the Beijerinckia or Azotobacter from the mixed culture on selective media.

Neither of these organisms has ever been isolated from any culture of Thiobacilli in this laboratory. Although Tsuchiya et al (1974) were able to grow a mixed culture of T.ferro oxidans and B.lacticogenes the latter had to be adapted to grow under the extreme acid conditions and to tolerate the toxic effect of high concentrations of dissolved ions found in leaching experiments. The growth of these organisms was estimated by a total count using a Coulter counter. These authors admit that counting was difficult but claim that in a 140 hours the mixed culture contained T.ferro oxidans  $8.6 \times 10^8$ /ml and B.lacticogenes  $5.0 \times 10^8$ /ml.

Our experiment on acetylene reduction has shown that if T.ferro oxidans is grown in a nitrogen-free medium but in an ammonia-rich atmosphere sufficient ammonium ions are absorbed for the nitrogenase to be repressed. In the situation described by Tsuchiya et al (1974) T.ferro oxidans would not need to fix nitrogen. B.lacticogenes would require a carbon source and I suggest it is more likely to have come from dead bacterial cells than from the  $\text{CO}_2$  fixed by T.ferro oxidans.

Although mixed Thiobacillus sp. may be present in some cultures the observed acetylene reduction by T.ferro oxidans in this laboratory was not caused by contamination with Beijerinckia or Azotobacter.

#### Confirmation by $^{15}\text{N}_2$ incorporation

Repression of acetylene reduction by growth of an organism with ammonium ions may be prima facie evidence for nitrogenase but the incorporation of gaseous  $^{15}\text{N}_2$  into the cellular material of the

bacterium is the only unequivocal proof of nitrogen fixation. To eliminate the possibility of a mixed culture the strain Tf3 was purified by a modification of the slide chamber technique (Postgate, Crumpton and Hunter, 1961) described in Section II. A 5cm petri dish containing 2.5ml of 'F' agarose medium was placed in a metal holder which could be used on an ordinary microscope stage. Lugs on the holder were aligned with reference marks on the plate to ensure that the plate could be relocated precisely if movement occurred during incubation. A plate in its holder is shown in Plate 12. The plates were inoculated with a dilute suspension of a log:phase culture of T.ferro oxidans Tf3 and the position of single bacterial cells identified by vernier scale readings. The plates, in their holders, were put inside larger petri dishes and these placed in a glass anaerobic jar under  $N_2 + 5\%CO_2$ . After 48 hours incubation the plates were observed microscopically and the positions of cells which had formed microcolonies were noted. The plates were re-incubated under the same conditions for 7 days during which the microcolonies grew into visible colonies. A deep brown precipitate of ferric sulphate within and around the colony showed that the organism was T.ferro oxidans. Cells from one of these colonies were picked and inoculated into a flask of liquid 'F' medium and the culture incubated at  $30^{\circ}C$  for 5 days. The whole procedure was repeated with this culture and the final strain considered pure, Tf3p.

The inoculum for the nitrogen-fixing experiment was prepared in a 'low phosphate' medium (D. Herbert personal communication) to reduce the loss of bacteria on the normal precipitate. The recipe is given in 'Methods and Materials'. A 3-day litre culture of Tf3p grown in 'F' medium was the seed for a bottle containing 20 litres of

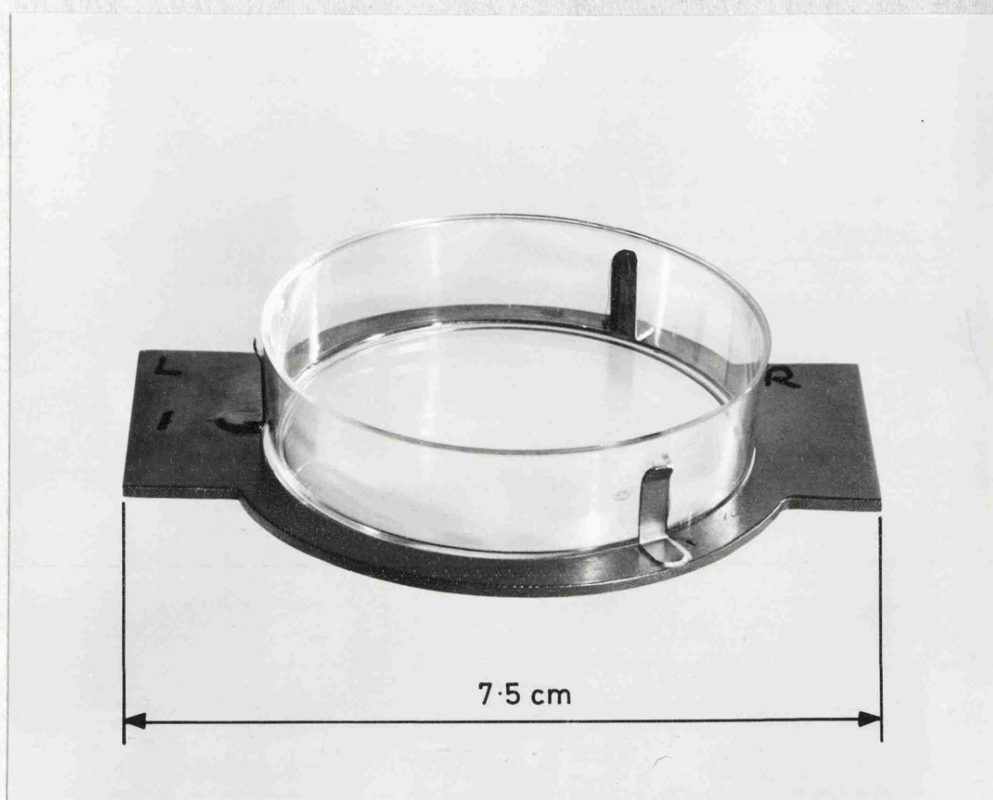


PLATE 12

Petri dish (5.5cm) in metal holder used for isolation  
of bacterial colonies arising from single bacterial cells  
of *T.ferro oxidans*

low phosphate medium. This culture was incubated at 30°C with forced aeration through a sintered glass sparger at 2 litres/minute for 48 hours. The culture was harvested by continuous flow centrifugation at 47,000g at 100ml/minute. The final yield of cells (about 0.5gms) was re-suspended in 0.005M H<sub>2</sub>SO<sub>4</sub>.

The incorporation experiments were carried out at room temperature in Bellco jars (A.R. Horwell, Kilburn High Road, London) which have a hanging bar magnet stirrer and two side arms which could be loosely covered with a screw cap, to prevent contamination but allow free passage of air, or hermetically sealed with 'Suba-seals'. A jar is shown in Plate 13. The jars were sterilised by autoclaving. A series of 5 jars was prepared each containing 210ml N-free F medium. Acetylene reduction test had indicated that the presumptive nitrogen-fixing system in T. ferro oxidans was like others, oxygen sensitive and that the best results were obtained with cells in the logarithmic phase of growth. T. ferro oxidans requires oxygen for growth and sufficient oxygen must be supplied to the culture to allow growth for several generations but insufficient to inhibit the expression of the nitrogenase system.

Because the acetylene reduction experiments had not established the optimum oxygen requirement the five culture jars were prepared and each was treated differently (Table 13). The <sup>15</sup>N<sub>2</sub> isotope was 99.4% pure (British Oxygen Company Ltd.). It was transferred by syringe from its sealed (suba-seal) container. The gas removed was replaced with an equal volume of sterile distilled water. All the jars were inoculated with 1.5ml of the prepared cells and were supplemented with 10ml FeSO<sub>4</sub> (30% w/v) after 18 hours,

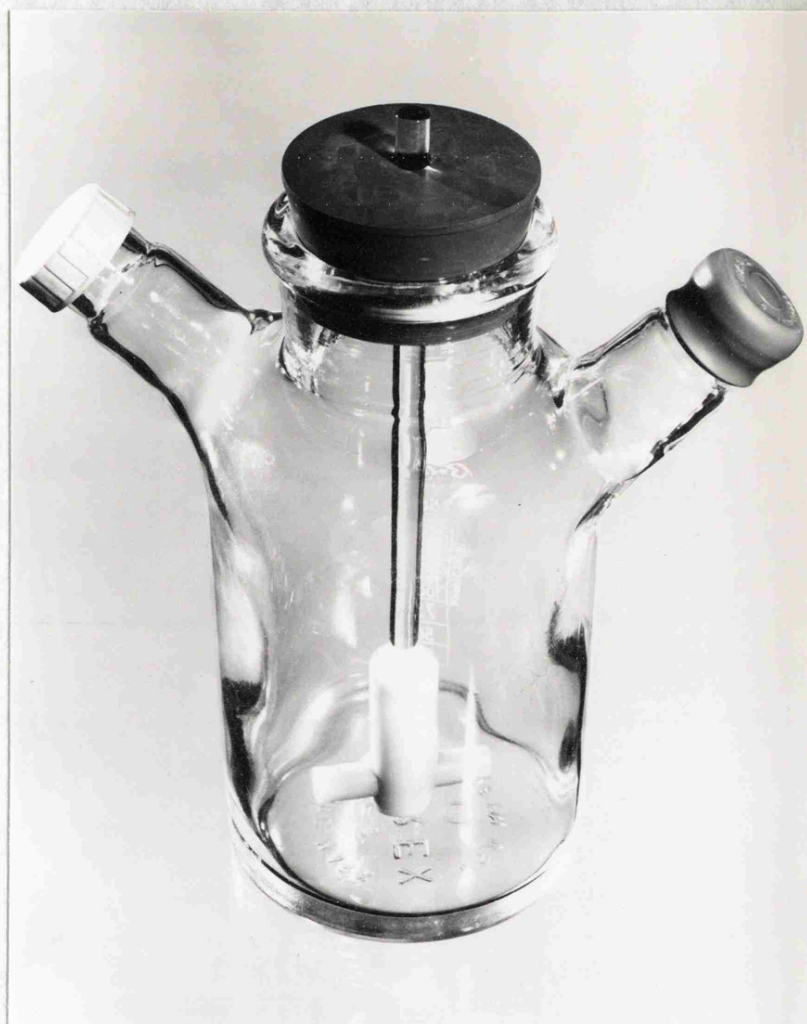


PLATE 13

Bellco jar used as the culture vessel for the incorporation of  $^{15}\text{N}_2$  into cell material. The left-hand side arm is capped and the right-hand side arm is sealed with a Suba-seal.

( $\frac{1}{2}$  actual size)

and a further 10 ml after 42 hours incubation. Two jars were sealed immediately and received 10ml  $^{15}\text{N}_2$ , the others were left unsealed for the first 18 hours. Because of the surface area to volume ratio of the culture and the high cell density of the inoculum ammonium ion absorption did not affect the enzyme system. After 18 hours the three remaining jars were sealed; two received 10ml  $^{15}\text{N}_2$  and the remaining jar received 10ml  $^{14}\text{N}_2$ . Of the two jars which had been sealed at the beginning of the experiment and received  $^{15}\text{N}_2$ , both received a further 10ml  $^{15}\text{N}_2$  and one received 10ml ammonia-free air and a further 10ml air at 42 hours. Jars which had originally received only 10ml  $^{15}\text{N}_2$  received a further 10ml at 42 hours and the  $^{14}\text{N}_2$  jar received a further 10ml at this time. After 4 days incubation at room temperature the contents of each jar was reduced to approximately 20ml by rotary evaporation under reduced pressure.

The nitrogen in the concentrated samples was determined at the ARC Unit of Nitrogen Fixation. E. Kavanagh carried out the digestions of the bacterial cells by conventional Kjeldahl and the Markham distillations. The gas samples of  $\text{N}_2$  were analysed on an AE1 MS 10 mass spectrometer by R.L. Richards who also interpreted the results of the mass spectrograms.

The natural abundance of  $^{15}\text{N}_2$  in free and combined  $\text{N}_2$  is 0.366 atoms %. If incubation with  $^{15}\text{N}_2$  leads to an enrichment under the conditions of the experiment then fixation of gaseous nitrogen has occurred. The results (Table 14) show that of the 4 jars to which  $^{15}\text{N}_2$  was added 3 gave a positive result. The other was sealed at the beginning of the experiment, was not supplied with any additional air during incubation and was presumed unable to obtain



sufficient  $O_2$  for growth and protein synthesis. A pure culture of T.ferro oxidans Tf3p was able to incorporate gaseous  $^{15}N_2$  into its cell protein and should therefore be considered a true nitrogen-fixer.

TABLE 13

Addition to cultures during incubation for  $^{15}N_2$  incorporation.

Time	Jar number				
	1	2	3	4	5
0				$^{15}N_2$	$^{15}N_2$
18	$^{14}N_2$	$^{15}N_2$	$^{15}N_2$	$^{15}N_2$	$^{15}N_2$
42	$^{14}N_2$	$^{15}N_2$	$^{15}N_2$	AIR	-
				AIR	-

All jars received 15ml (30% w/v)  $FeSO_4$  at 18 hours and 10ml(30% w/v)  $FeSO_4$  at 42 hours. The volumes of all components added was 10ml unless stated otherwise.

TABLE 14

Fixation of  $N_2$  by T.ferro oxidans

Jar number	atom % $^{15}N_2$ from cells in culture	accuracy $\pm$	Atom % increase over the natural abundance
1	0.360	-	-
2	0.479	-	0.113
3	0.421	0.007	0.055
4	0.518	0.015	0.152
5	0.369	-	0.003

#### Ability among strains of *T.ferro oxidans* to fix nitrogen

The acetylene reduction test was used to establish the extent of the nitrogen-fixing ability among strains of *T.ferro oxidans* and showed that all strains tested reduced acetylene to ethylene with similar efficiencies to Tf3p. If T.pyrite (10% w/v) was supplied as the source of energy in such an experiment acetylene reduction occurred but the time required was much longer. Tf3 gave an ethylene peak height of 63 x 100mm after 21 days incubation. Unfortunately the efficient pyrite utilising strains Tf52 and Tf74 were not tested. Although ELO (20% w/v) was tried as a substrate no evidence of acetylene reduction was found in 60 days. However, growth of *T.ferro oxidans* is slow on ELO and in 60 days growth of a sufficient cell density to reduce a detectable quantity of ethylene may not have been achieved.

*T. ferro oxidans* is able to grow using sulphur as its source of energy and grows well on 'F' basal salts pH3.5 + 1%(w/v) powdered sulphur. An experiment with Tf3 using an inoculum culture that was sulphur grown showed that in an acetylene reduction experiment in N-free F salts pH3.5 ethylene was not produced when sulphur was present in the medium either as the sole energy source or with ferrous sulphate. Sulphur grown Tf3 in N-free F salts pH3.5 with ferrous sulphate alone produced an ethylene peak of 140 x 100mm in 2 days. This observation that sulphur inhibits acetylene reduction requires further study.

#### Summary of nitrogen fixation by *T.ferro oxidans*

The ability of a pure culture of *T.ferro oxidans* to fix atmospheric nitrogen was proved by the incorporation of  $^{15}\text{N}_2$  into the bacterial cell material. The observed acetylene reduction by *T.ferro oxidans* can therefore be presumed to be due to a nitrogenase

enzyme system. This enzyme is very sensitive to the ammonium ion concentration of the medium and can be repressed by absorption of ammonia from the atmosphere into the acid medium.

All strains of T.ferro oxidans in the culture collection were shown by acetylene reduction to have a nitrogenase and the possibility that the results obtained were due to contamination with known nitrogen fixers was excluded as no strain of T.ferro oxidans lost its acetylene reducing ability on purification and no contaminant, which could fix nitrogen, was ever isolated.

Sulphur appeared to inhibit acetylene reduction. Why should T.ferro oxidans be able to fix atmospheric nitrogen? These acidophilic organisms flourish in their ecological niche because they fix carbon dioxide from the air and do not require an organic carbon source, they utilise the metal sulphides for their energy and produce sulphuric acid and ferric sulphate which help to liberate a further source of energy and their oxygen requirement is small. The natural environment of pyritic ores would not be associated with an ammonia rich atmosphere as few other organisms live in the highly acidic environment and decomposition of dead material would be minimal. If pyritic and metal sulphide ores are, like Tharsis pyrite, deficient in soluble nitrogen the ability to fix nitrogen from the atmosphere would be an evolutionary advantage.

SECTION VIMethods and Materials

Details of media, techniques and equipment not mentioned in the text are given in this section.

Media in general useFerrous sulphate medium 'F' (Leathen, Kinsel and Braley, 1956)

## basal salts

$(\text{NH}_4)_2 \text{SO}_4$	0.15 gms
KCl	0.05 gms
$\text{KH}_2 \text{PO}_4$	0.05 gms
$\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$	0.50 gms
$\text{Ca}(\text{NO}_3)_2$	0.01 gms
$\text{H}_2\text{O}$	1000 ml

pH 2.2                      autoclaved at 15 lb for 15 minutes

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was made up at 30% w/v in 0.005M  $\text{H}_2\text{SO}_4$  and sterilised by Millipore filtration. The sterile solution was dispensed in 20ml amounts into sterile 1oz plastic bottles and stored at  $-20^\circ\text{C}$ . It was thawed at room temperature and added to the 'F' basal salts at the required concentration to give 'F' medium.

$$30\% \text{FeSO}_4 \cdot 7\text{H}_2\text{O} \equiv 300 \text{ mg/ml} \equiv 60.26 \text{ mg/ml Fe}$$

For use with solidifying agents the basal salts were made at double strength and mixed 1 : 1 with the molten agent. Ferrous sulphate was then added as required.

Sulphur medium 'S' (Hutchinson, Johnstone and White, 1965)

## basal salts

$\text{Na}_2 \text{H PO}_4$	1.2 gms
$\text{K H}_2 \text{ PO}_4$	1.8 gms
$\text{Mg SO}_4 \cdot 7\text{H}_2\text{O}$	0.1 gms
$(\text{NH}_4)_2 \text{ SO}_4$	0.1 gms
$\text{Ca Cl}_2$	0.03 gms
$\text{Fe Cl}_3 \cdot 6\text{H}_2\text{O}$	0.02 gms
$\text{Mn SO}_4 \cdot 4\text{H}_2\text{O}$	0.02 gms
$\text{H}_2\text{O}$	1000 ml
pH 3.5	autoclaved at 15 lb for 15 minutes.

Sulphur. Precipitated sulphur (BDH) was put in 1.0 gm amounts in  $\frac{1}{4}$ oz bijou bottles and steam sterilised for 30 minutes on three consecutive days. Sulphur was added in excess to the basal salts; approx. 1gm to 200 ml.

Thiosulphate medium 'ST' (Hutchinson, Johnstone and White 1965)

Basal salts as for 'S' medium with addition of

$\text{Na}_2 \text{ S}_2 \text{ O}_3 \cdot 5\text{H}_2\text{O}$  10 gms/litre

pH 5.5 autoclaved at 15 lb for 15 minutes.

For use with solidifying agents the medium was made at double strength and mixed 1 : 1 with the molten agent.

Diluent

0.005 M  $\text{H}_2\text{SO}_4$  This can be prepared either by adding 0.5 ml of 1M  $\text{H}_2\text{SO}_4$  to 1000 ml  $\text{H}_2\text{O}$  or by adding  $\text{H}_2\text{SO}_4$  to water until the pH is 2.1. Autoclaved at 15 lb for 15 minutes.

### Requirements for growth on solid media

#### Silica gel plates (Meiklejohn, 1950)

Sodium silicate (BDH) solution was prepared according to Meiklejohn and the technique described by her for the preparation of plates followed with the exception that Leathen's 'F' medium was used as the mineral salts.

#### Agarose plates

Agarose (BDH)	1.6 gms
H <sub>2</sub> O	100 ml

The agarose was dissolved by steaming for 1 hour. It was dispensed either as 50 ml amounts in 4oz bottles or 5 ml in 1oz bottles and autoclaved at 15 lb for 15 minutes.

#### Agar plates

Agar	2.0 gms
H <sub>2</sub> O	100 ml

All agar suspensions, irrespective of brand, were made up at 2% w/v and dissolved by steaming for 1 hour. They were dispensed in 50 ml amounts in 4oz bottles or 5 ml in 1oz bottles and autoclaved at 15 lb for 15 minutes.

Gas and gas mixtures were supplied by the British Oxygen Company,

Deer Park Road, London S.W.19

N<sub>2</sub> + 5% CO<sub>2</sub>

Air + 5% CO<sub>2</sub>

Argon - high purity

Hydrogen - high purity

Nitrogen - oxygen free (white spot)

### Anaerobic jars

Supplied by Gallenkamp. Heavy-walled glass jar of modified McIntosh and Fields pattern. CX500.

A. Gallenkamp & Co.Ltd., Technico House, Christopher Street,  
London E.C.2.

### Slide culture chambers

The annuli for the slide culture chambers were made in the M.R.E. workshops to a specification of 20mm internal diameter, 22 mm external diameter and 1mm depth. They were made of brass or stainless steel.

### Plating techniques

The techniques for 'spread' plates and 'pour' plates are those given by Meynell and Meynell (1965). Chapter 2 of this book describes the bacteriological techniques used including total cell counts using Helber chambers.

### Requirement for growth in liquid media

Iron determination in culture of *T.ferro oxidans* growing in ferrous sulphate media (D. Herbert - personal communication). This is a colorimetric determination of  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  as the yellow  $\text{FeCl}_3\text{-HCl}$  complex. The colour is very sensitive to HCl concentration which should be the same in both unknowns and standards. The 6N.HCl was made up in a "winchester", enough being made for use throughout a growth experiment.

Reagents

HCl (Analar concentrated)  
 6N•HCl (Analar)  
 1M H<sub>2</sub>O<sub>2</sub> in 6N•HCl (prepared fresh daily)  
 Stock iron ammonium alum, 1mg Fe<sup>3+</sup>/ml  
 Working standard 50 µg Fe<sup>3+</sup>/ml in 6N•HCl

For the stock solution iron ammonium alum was dissolved

8.633 gm (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>•Fe<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub>•24H<sub>2</sub>O in 1 litre 0.1 N•H<sub>2</sub>SO<sub>4</sub>

For the working standard to 10 ml stock iron ammonium alum was added 10 ml con<sup>c</sup>HCl and 6N•HCl to a volume of 200 ml.

Ferric iron in solution was determined by direct colorimetry of the FeCl<sub>3</sub> • HCl complex and total iron (Fe<sup>2+</sup> + Fe<sup>3+</sup>) was similarly determined after adding H<sub>2</sub>O<sub>2</sub> to oxidise all the ferrous to ferric.

Method A 2ml sample of culture was mixed on a 'Whirlimixer' to break up any particles of precipitate; 1.0ml was immediately measured into a 10ml graduated tube. 1.0ml of con<sup>c</sup>HCl was added by burette and the content mixed on the 'Whirlimixer'. The solution was then made up to 10ml with 6N•HCl. 0.5ml portions of this solution were measured into two 10ml graduated tubes; one of these (a) was diluted to 10ml with 6N•HCl; the other (b) was treated with 0.1ml of 1M•H<sub>2</sub>O<sub>2</sub> in 6N•HCl, shaken for 30 seconds and then diluted to 10ml with 6N•HCl. Tubes (a) and (b) together with a tube containing 5ml of the working standard Fe<sup>3+</sup> solution, were incubated in a waterbath at room temperature for 10 minutes and the absorption measured on a Pye Unicam SP600 spectrophotometer against 6N•HCl as a blank, using 1cm cells and a wavelength of 431.5 nm.



### Calculation

$$\mu\text{g Fe}^{3+} \text{ in sample} = 500 \times \frac{\text{absorbance of sample}}{\text{absorbance of standard}}$$

tube 'a' gave amount of ferric iron in solution

tube 'b' gave amount of total iron in solution.

### Modification of iron estimation for growth on pyrite and pyritic ores

The initial 2ml sample was centrifuged at 5000 rpm for 5 minutes to deposit pyritic material, 1.0 ml of the supernatant was measured into a 10ml graduated tube. The procedure was then as for ferrous sulphate media.

### Incubator

The incubator used for the growth studies was a New Brunswick, Psychrotherm-controlled environment, orbital, incubator shaker G.26 0-60°C, New Brunswick Scientific Co.Inc., New Brunswick N.J., U.S.A.

### Aagate Ball Mill

The mill was obtained from Glencreston & Co.Ltd.,  
37 The Broadway, Stanmore, Middlesex.

### Standard Sieves

The milled pyrite and pyritic ores were sieved to the required mesh size in a set of standard sieves obtained from A. Gallenkamp & Co.Ltd., Technico House, Christopher Street, London E.C.2.

Requirements for nitrogen-fixation

A nitrogen-free modification of Leathen's medium was used for all nitrogen-fixing experiments.

N-free medium

KCl	0.05 gms
$\text{KH}_2\text{PO}_4$	0.05 gms
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.50 gms
$\text{CaCl}_2$	0.02 gms
$\text{Na}_2 \text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.02 gms
$\text{H}_2\text{O}$	1000 ml
pH 2.2	autoclaved at 15 lb for 15 minutes

The medium was used with ferrous sulphate which was prepared as described in the subsection on media for general use.

Low phosphate medium for growth of inoculum

The low phosphate medium was devised by D. Herbert (personal communication).

low-phosphate salts x 100

$(\text{NH}_4)_2\text{SO}_4$	13.2 gms
$\text{KH}_2\text{PO}_4$	2.72 gms
$\text{H}_2\text{O}$	1000 ml
pH 2.2 with $\text{H}_2\text{SO}_4$	autoclaved at 15 lb for 15 minutes

Trace element mixture (Salts 22)

Con <sup>c</sup> HCl	250 ml
MgO	50.4 gms
CaCO <sub>3</sub>	1.0 gm
FeCl <sub>3</sub> • 6H <sub>2</sub> O	2.7 gms
Mn Cl <sub>2</sub> • 4H <sub>2</sub> O	0.99 gms
Zn O	0.41 gms
CoCl <sub>2</sub> • 6H <sub>2</sub> O	1.19 gms
H <sub>3</sub> BO <sub>3</sub>	0.31 gms
Na <sub>2</sub> MoO <sub>4</sub> • 2H <sub>2</sub> O	1.21 gms
CuCl <sub>2</sub> • 2H <sub>2</sub> O	0.34 gms
H <sub>2</sub> O	to make 1000 ml

Use 1 ml per litre of medium. The concentrated solution may be stored indefinitely at room temperature without sterilisation.

For use in media the salts are sterilised by Millipore filtration and added aseptically.

Low-phosphate medium

0.005M H <sub>2</sub> SO <sub>4</sub> (sterile)	900 ml
low phosphate salts x 100	10 ml
Salts 22	1 ml
FeSO <sub>4</sub> • 7H <sub>2</sub> O 30% w/v	90 ml (or as required)

Acetylene

Acetylene was prepared from calcium carbide (BDH) and water as described by Postgate (1972).

### Ethylene

Ethylene 99.9% pure was obtained from B.O.C.

### Isotopic nitrogen

The isotope  $^{15}\text{N}_2$  was obtained from B.O.C. at 99.4% enrichment.

### Bellco jars

The confirmation growth experiments for  $^{15}\text{N}_2$  incorporation were carried out in Bellco jars which were obtained from A.R. Horwell, Kilburn High Road, London.

### Suba-seal closures

Rubber Suba-seal closures were obtained from William Freeman Co.Ltd. Staincross, Barnsley, Yorkshire.

### Gas-liquid chromatography

Gas-liquid chromatography was run on a Pye Unicam model 104 chromatograph with a hydrogen flame ionisation detector at  $130^\circ\text{C}$  and a 152 cm glass column packed with 'Porapak R' held at  $65^\circ\text{C}$ . The carrier gas was nitrogen at a flow rate of 45ml/minute. Gas samples of 1ml were injected directly into the chromatograph through a gas sampling valve.

Pye Unicam Ltd., York Street, Cambridge CB1 2PX

'Porapak R' was obtained from Phase Separation Ltd., Deeside

Industrial Estate, Queensferry, Flint. CH5 ZLP

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